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(74) Agents: WELCH, Teresa, J. et al.; Michael Best &amp; Friedrich LLP, One South Pinckney Street, Suite 700, P.O.Box 1806, Madison, WI 53701-1806 (US).

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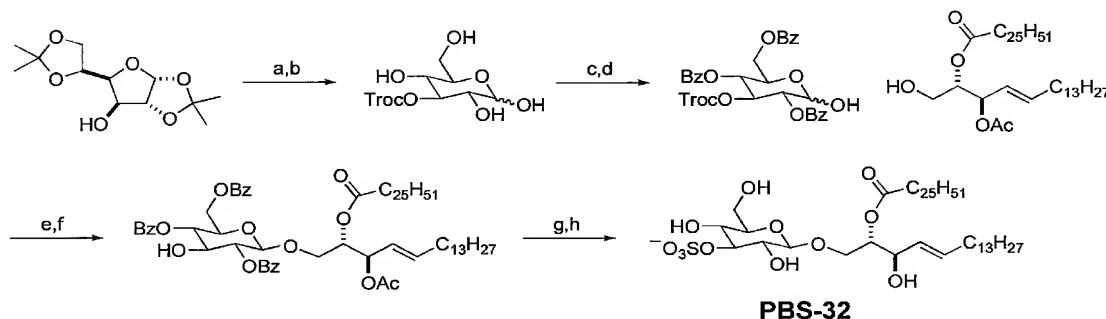
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(71) Applicants (for all designated States except US): THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US). THE UNIVERSITY OF CHICAGO [US/US]; 5801 South Ellis Avenue, Chicago, IL 60637 (US). BRIGHAM YOUNG UNIVERSITY [US/US]; 3760 HBLL, Provo, UT 84602-6844 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): TEYTON, Luc [US/US]; 14226 Pinewood Drive, Del Mar, CA 92014 (US). BENDELAC, Albert [US/US]; 1121 East 53rd Street, Chicago, IL 60615 (US). SAVAGE, Paul, B. [US/US]; 1075 East Maple Street, Mapleton, UT 84664 (US).

(54) Title: ADJUVANTS AND METHODS OF USE



(57) Abstract: Compositions comprising NKT cell agonist compounds and a physiologically acceptable vehicle are provided. Methods of stimulating an NKT cell and enhancing an immune response are also disclosed. Further provided are vaccine preparations comprising NKT cell agonist compounds.

WO 2008/005824 A1

## **ADJUVANTS AND METHODS OF USE**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

5        This application claims the benefit of U.S. Provisional Application No. 60/806,330 filed June 30, 2006. The provisional application is incorporated herein by reference.

### **STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH**

10        This invention was made with United States government support awarded by the National Institutes of Health, under grant number PO1 AI053725. The United States has certain rights in this invention.

### **BACKGROUND OF THE INVENTION**

#### **15        Field of the Invention**

      The invention relates generally to the field of immunobiology. In particular, the invention relates to compositions and methods useful in stimulating an NKT cell or enhancing an immune response against a disease or disorder.

#### **20        Introduction**

      Lipid species are ubiquitous constituents of all eukaryotic membranes and pathogenic microorganisms. Despite the abundance of lipids in biological systems, the anti-lipid immune response has not been studied to the same extent as the anti-peptide response. For example, comparatively little is known about the phenomenon 25 of non-toll-like receptor (non-TLR) recognition of lipid antigens. Unlike peptide antigens, lipids are processed and presented to the immune system by the CD1 family of  $\beta$ 2 microglobulin-associated molecules. In contrast to class I and II major histocompatibility complex (MHC) molecules that present peptide antigens to CD8+

and CD4+ T cells, respectively, CD1 molecules have evolved to capture and process both foreign and self lipid antigens for display to particular subsets of T cells.

A variety of lipids with different structures have been shown to bind CD1 molecules in a unique manner that accommodates a fatty acid chain in each of the 5 two hydrophobic binding pockets (A' and F) of the CD1 molecule. Lipid species capable of binding CD1 molecules include mycolic acids, diacylglycerols, sphingolipids, polyisoprenoids, lipopeptides, phosphomycoketides and small hydrophobic compounds.

The CD1 presentation pathway triggers both innate and adaptive immune 10 responses by activating two complementary CD1-restricted T cell subsets: NKT cells that perform adjuvant functions, and non-NKT T cells capable of helper or cytolytic functions.

NKT cells, which express both natural killer (NK) cell surface markers and a conserved, semi-invariant T-cell receptor (TCR), V $\alpha$ 14-J $\alpha$ 18/V $\beta$ 8 in mice and V $\alpha$ 24-15 J $\alpha$ 18/V $\beta$ 11 in humans, are characterized by self lipid reactivity and rapid effector responses. Accordingly, NKT cells play an important role in a number of immune functions, including antimicrobial responses, antitumor immunity and regulation of the balance between tolerance and autoimmunity.

The apparent pluripotency of NKT cells depends specifically on their ability to 20 interact with dendritic cells (DCs), to determine the T<sub>H1</sub> or T<sub>H2</sub> polarity of the T cell response, and to initiate T cell anergy in appropriate circumstances. The maturation and recruitment process of DCs is central to their function. Indeed, DCs can rapidly progress from quiescence and low metabolic activity to active uptake of antigen, processing and tissue migration after receiving differentiation signals.

25 A number of natural and synthetic lipid molecules are processed by DCs and presented by CD1 molecules to NKT cells. The prototypical compound used to study NKT cell activation *in vitro* and *in vivo* is KRN7000, an  $\alpha$ -galactosylceramide

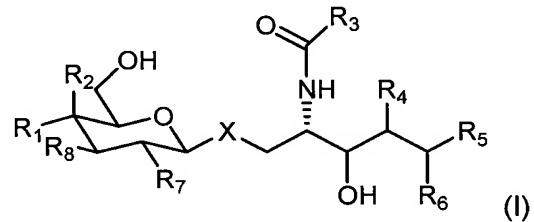
(“ $\alpha$ GalCer” or “PBS-57”) derived from marine sponge *Agelas mauritianus*. Additional agonists include isoglobotrihexosylceramide (“iGb3” or “PBS-47”), which is an endogenous glycosphingolipid, as well as members of a class of microbial-derived  $\alpha$ -glycuronosylceramides. However, very little has been elucidated regarding anti-lipid 5 responses in general. In the context of vaccination in particular, even less is known regarding the mechanism of lipid adjuvanticity.

Adjuvants are used to augment the immune response in anti-microbial and anti-tumor vaccination protocols, as well as in experimental immunology. The 10 chemical nature of adjuvants, their mechanisms of action and their side effect profiles are highly variable. In some cases, side effects can be ascribed to an inappropriate immune response, or in other cases, can be the result of adverse pharmacological reactions. At present, the choice of adjuvant for human vaccination reflects a compromise between the requirement for adjuvanticity and an acceptable level of side effects.

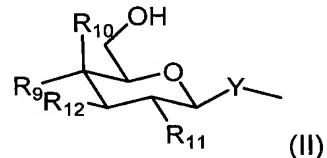
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### SUMMARY OF THE INVENTION

In one aspect, the invention provides a composition comprising a compound represented by structural formula (I):

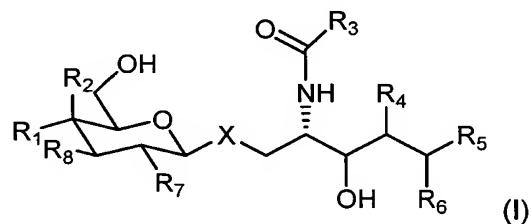


20 wherein: X is -O-, -CH<sub>2</sub>- or -S-; R<sub>1</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H, -COOH or a group represented by structural formula (II):

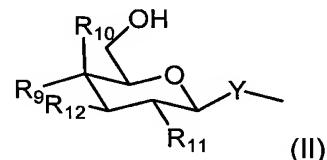


wherein: Y is -O-, -CH<sub>2</sub>- or -S-; R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>3</sub>H and -COOH; R<sub>2</sub> is selected from -H, OSO<sub>3</sub>H, -SO<sub>3</sub>H and -PO<sub>4</sub>; R<sub>7</sub> and R<sub>8</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH; R<sub>3</sub> is a saturated or unsaturated hydrocarbon group having about 7 to about 25 carbon atoms; R<sub>4</sub> is -H, -OH, or, together with R<sub>6</sub>, forms a carbon-carbon double bond; R<sub>5</sub> is a saturated or unsaturated hydrocarbon group having from about 5 to about 15 carbon atoms, and R<sub>6</sub> is -H, -OH, or together with R<sub>4</sub> forms a C-C double bond, provided that at least one of R<sub>1</sub>, R<sub>2</sub>, R<sub>7</sub>, R<sub>8</sub>, R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> or R<sub>12</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H or -COOH; and a physiologically acceptable vehicle.

In another aspect, the invention provides a composition comprising a compound represented by structural formula (I):



wherein: X is -O-, -CH<sub>2</sub>- or -S-; R<sub>1</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H, -COOH or a group represented by structural formula (II):



wherein: Y is -O-, -CH<sub>2</sub>- or -S-; R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>3</sub>H and -COOH; R<sub>2</sub> is selected from -H, -OH, OSO<sub>3</sub>H, -SO<sub>3</sub>H and -PO<sub>4</sub>; R<sub>3</sub> is a saturated or unsaturated hydrocarbon group having from

about 7 to about 25 carbon atoms; R<sub>4</sub> is -H, -OH, or, together with R<sub>6</sub>, forms a carbon-carbon double bond;

R<sub>5</sub> is a saturated or unsaturated hydrocarbon group having from about 5 to about 15

carbon atoms; R<sub>6</sub> is -H, -OH, or together with R<sub>4</sub> forms a C-C double bond; R<sub>7</sub> is

5 selected from -H, -OH, -OSO<sub>3</sub>H, SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH; and R<sub>8</sub> is selected from -H, -OH, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH; provided that at least one of R<sub>1</sub>, R<sub>2</sub>, R<sub>7</sub>, R<sub>8</sub>, R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> or R<sub>12</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H or -COOH; and a physiologically acceptable vehicle.

10 In some embodiments, the composition optionally includes an antigen. Some embodiments of the invention provide the composition formulated as a vaccine preparation.

In a further aspect, the invention provides a method of stimulating an NKT cell comprising contacting the NKT cell with a compound having a structural formula 15 represented by formula (I). In some embodiments, the NKT cell is cultured *in vitro*, while in other embodiments, the NKT cell is within a subject, or "*in vivo*."

In another aspect, the invention provides a method of enhancing an immune response in a subject. The method includes administering the compound of formula 20 (I) to the subject. In some embodiments, the compound of formula (I) is co-administered with an antigen.

In a still further aspect, the invention provides a vaccine preparation, formulated to include a compound of formula (I) and a physiologically acceptable vehicle.

25

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts a suitable synthetic scheme for sulfoglucosylceramide (SuGC), also referred to herein as PBS-32.

FIG. 2 depicts the structural formulae for several compounds referenced herein, including PBS-32 and modified ceramide compounds designated PBS-31 (phytoceramide) PBS-46, PBS-41 (sulfatide), PBS-36, PBS-58, PBS-35 and PBS-40.

FIG. 3 is a photograph of an isoelectric focusing gel showing loading of CD1d 5 molecules with PBS-31, PBS-32, PBS-41, PBS-46, PBS-36, PBS-58, PBS-35 and PBS-40 in the presence and absence of saposin B, a lipid transfer glycoprotein.

FIG. 4 is a graph showing differential activation and dose responsiveness of Va14 NKT cell hybridoma DN32.D3 by PBS-32, PBS-31,  $\alpha$ GalCer and vehicle.

FIG. 5 is a graph showing results of enzyme-linked immunosorbant assays 10 (ELISAs) measuring IFN- $\gamma$ , IL-10, IL-4 and GM-CSF production following 24 hour stimulation of splenocytes with PBS-47, PBS-32, PBS-31 and  $\alpha$ GalCer.

FIG. 6 is a graph showing the results of surface plasmon resonance (SPR) measurements of the binding of PBS-32-CD1d complexes to recombinant Va14/2C $\beta$  TCR.

15 FIG. 7 is a graph showing activation of a human NKT cell line by homologous DCs pulsed with decreasing concentrations of  $\alpha$ GalCer, PBS-47, PBS-32 and PBS-31. Activation of NKT cells was measured by ELISA for IFN- $\gamma$  and IL-4.

FIG. 8 is a graph showing results of fluorescence activated cell sorting 20 (FACS) analysis for cell surface expression of CD40, CD80, CD86 and MHC class II by splenic DCs (CD11c+, CD8a+ and CD11c+/CD8a-) 24 hours after injection with PBS-31, PBS-32,  $\alpha$ GalCer and vehicle.

FIG. 9 graphically represents the ability of PBS-32 to act as an adjuvant when administered with an ovalbumin nominal antigen. FIG. 9A shows percentages of H-2K $b$ /ova<sub>257-264</sub> responsive CD8+ T-cells (as indicated by tetramer staining and FACS 25 analysis) after administration of vehicle alone, OVA alone, OVA +  $\alpha$ GalCer (PBS-57) and OVA + PBS-32. FIG. 9B shows relative amounts of anti-ovalbumin IgG antibody, as measured by ELISA.

FIG. 10 is a graph showing results of FACS staining for IFN- $\gamma$ -producing CD8+ and CD4+ T-cells after a five hour *in vitro* pulse with LCMV antigens and antigen presenting cells.

FIG. 11 is a graph showing IgM and IgG anti-GM3 antibody concentration, as measured by ELISA, after biweekly immunization with GM3 in combination with a lipid transfer protein (either LBP, CD14, ApoH, NPC-2, GM2A or saposin B) and  $\alpha$ GalCer. Each bar represents a separate bleed (every other week). FIG.11A is a graph of antibody concentrations using of hGM2a and mNPC2, FIG. 11B is the graph of antibody concentrations using mSapB and mCD14, and FIG. 11C is the graph of antibody concentrations using hLBP and hApoH.

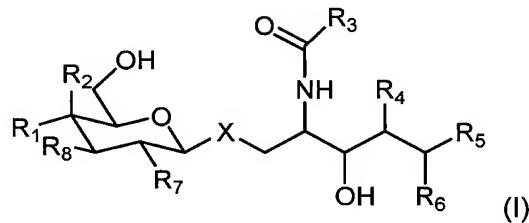
FIG. 12 is a graph showing specificity of anti-GM3 monoclonal antibodies produced by mice immunized with lipids as measured by ELISA and FACS analysis of liposome-coated glass beads.

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## DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The inventors have discovered that an endogenous sulfoglucosylceramide, designated "PBS-32," and its phytoceramide counterpart, designated "PBS-31," are strong agonists of NKT cells. As such, these compounds can enhance an immune response in a subject under appropriate circumstances. Because they are endogenous compounds, the likelihood of side effects is reduced in comparison to exogenous adjuvants. Similarly, variants of these compounds that have been modified, e.g., to introduce properties suitable for *in vivo* delivery, are also expected to exhibit a reduced likelihood of side effects. As further described below, suitable variants include modifications of the sulfate group, the ceramide head group, the sugar linkage, the fatty acid and the sphingosine side chain of PBS-32. PBS-32 and variants of this compound that exhibit NKT cell agonist activity are collectively referred to herein as "NKT cell agonist compounds."

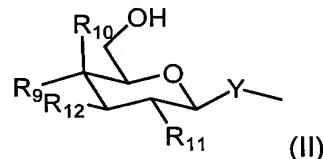
NKT cell agonist compounds have a structure represented by formula (I):



wherein:

5            X is -O-, -CH<sub>2</sub>- or -S-;

          R<sub>1</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>3</sub>H, -COOH or a group  
          represented by structural formula (II):



wherein:

10           Y is -O-, -CH<sub>2</sub>- or -S-;

          R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are independently selected from -H, -OH, -  
          OSO<sub>3</sub>H,  
          -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>3</sub>H and -COOH;

          R<sub>2</sub>, R<sub>7</sub> and R<sub>8</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H -  
15           SO<sub>3</sub>H, -PO<sub>4</sub>,  
          -PO<sub>4</sub>H, and -COOH;

          R<sub>3</sub> is a saturated or unsaturated hydrocarbon group having about 7 to  
          about 25 carbon atoms;

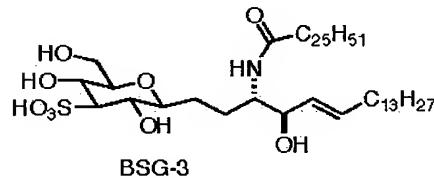
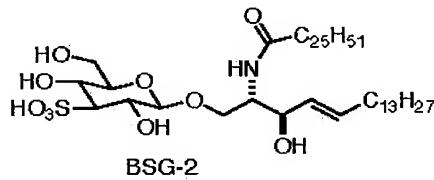
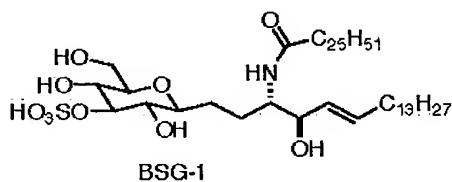
          R<sub>4</sub> is -H, -OH, or, together with R<sub>6</sub>, forms a carbon-carbon double  
20           bond; and

          R<sub>5</sub> is a saturated or unsaturated hydrocarbon group having about 5 to  
          about 15 carbon atoms,

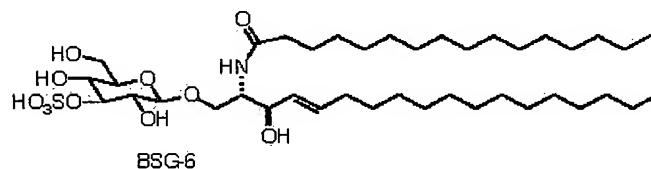
provided that at least one of  $R_1$ ,  $R_2$ ,  $R_7$ ,  $R_8$ ,  $R_9$ ,  $R_{10}$ ,  $R_{11}$  or  $R_{12}$  is -H, -OH, - $OSO_3H$ , - $SO_3H$ , - $PO_4$ , - $PO_4H$ , or - $COOH$ .

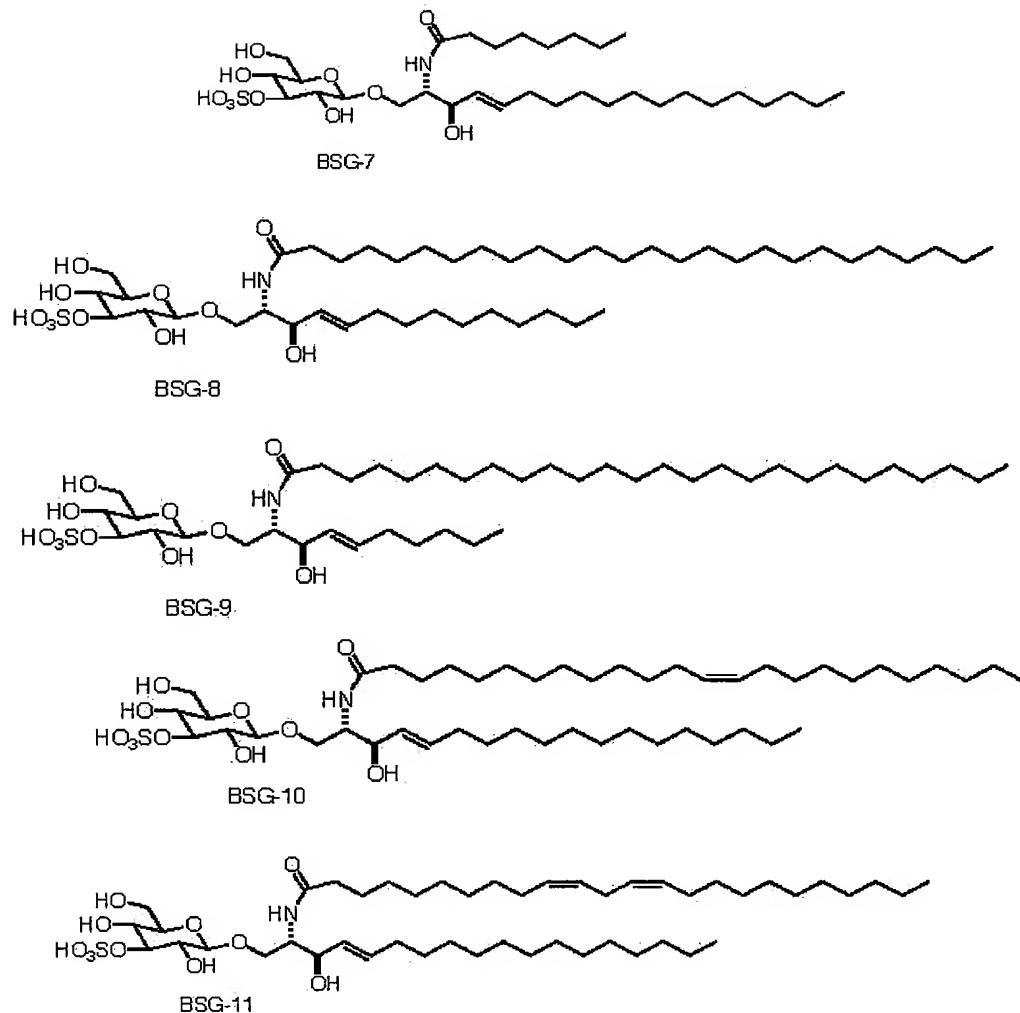
As used herein, the term "saturated or unsaturated hydrocarbon" refers to  
 5 straight-chain alkyl or alkenyl groups of specified lengths. Alkenyl groups include  
 one or more double bonds. Suitably, alkenyl groups of compounds of formula (I) may  
 include from about 1 to about 3 carbon-carbon double bonds.

Suitable NKT cell agonist compounds may include, but are not limited to,  
 PBS-32 and PBS-31. Non-limiting examples of further suitable NKT cell agonist  
 10 compounds having a modified glycoside bond and/or sulfate group are shown below:



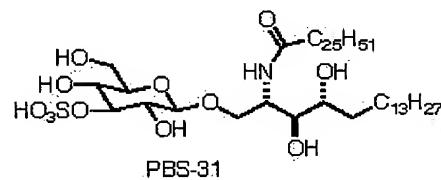
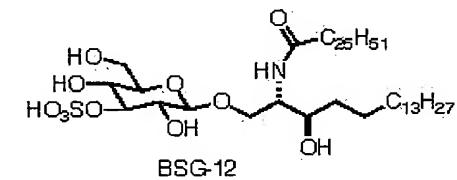
15 Non-limiting examples of suitable NKT cell agonist compounds having  
 modified lipid chains are shown below:

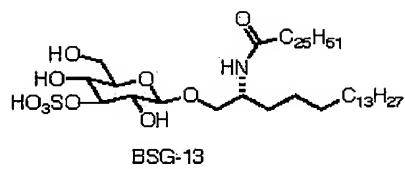




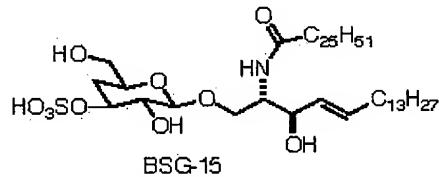
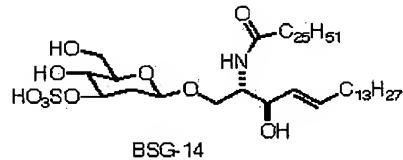
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Non-limiting examples of suitable NKT cell agonist compounds having modified ceramide chains are shown below:

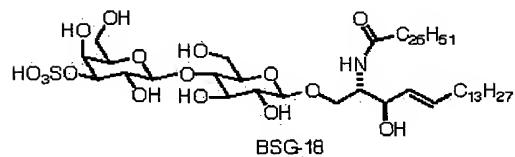
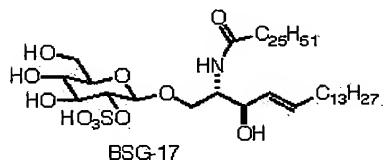
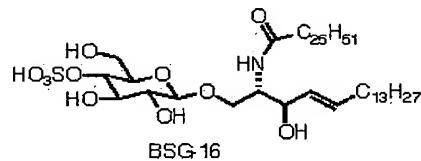




Non-limiting examples of suitable NKT cell agonist compounds having modified carbohydrate groups are shown below:



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Endogenous NKT cell agonist compounds may be purified from natural sources  
 10 using any suitable method known in the art. Alternatively, the NKT cell agonist compounds may be chemically synthesized. One suitable scheme for synthesis of NKT cell agonist compounds is described in Example 1, below. Variant compounds

may be synthesized by modifications of the method, as may be derived from Zhou, D et al., *Science* 306: 1786-1789 (2004); Goff, R. D. et al., *J. Am. Chem. Soc.* 126: 13602-13603 (2004); and Marshall, R. L. et al. *Tetrahedron Lett.*, 39: 3923-3926 (1998), each of which are incorporated herein by reference in their entireties.

5

### Compositions

NKT cell agonist compounds, as described above, are suitably included in a composition with a physiologically acceptable vehicle. A "physiologically acceptable" vehicle is any vehicle that is suitable for *in vivo* administration (e.g., oral, transdermal 10 or parenteral administration) or *in vitro* use, i.e., cell culture. Suitable physiologically acceptable vehicles for *in vivo* administration include water, buffered solutions and glucose solutions, among others. A suitable vehicle for cell culture is commercially available cell media. Additional components of the compositions may suitably include excipients such as stabilizers, preservatives, diluents, emulsifiers or 15 lubricants, in addition to the physiologically acceptable vehicle and the NKT cell agonist compound. In particular, suitable excipients include, but are not limited to, Tween 20, DMSO, sucrose, L-histidine, polysorbate 20 and serum.

Suitably, compositions comprising NKT cell agonist compounds may be formulated for *in vivo* use, i.e., therapeutic or prophylactic administration to a subject. 20 In some embodiments, the compositions are formulated for parenteral administration. A suitable dosage form for parenteral administration is an injectable. An injectable dosage form may be an isotonic solution or suspension and may be prepared using a suitable dispersion agent, wetting agent or suspension agent, as known in the art. In other embodiments, the compositions are formulated for oral administration. Suitable 25 oral dosage forms include tablets, capsules, syrups, troches and wafers, among others. Oral dosage formulations suitably include lactose, starch, cellulose derivatives, magnesium stearate, stearic acid, glycols, and others. It will be

appreciated that the compositions of the invention are not limited to any particular exemplified dosage form, but can be formulated in any manner described in the art, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co., (2000), which is incorporated herein by reference.

5        In addition to the NKT cell agonist compound and a physiologically acceptable vehicle, some embodiments of the invention further include an antigen and are suitably formulated as a vaccine preparation. Antigens included in the vaccine preparation may be polypeptide or carbohydrate moieties, or combinations thereof, for example, glycoproteins. The antigen may be derived from an infectious 10 agent (e.g., a pathogenic microorganism), a tumor, an endogenous molecule (e.g., a "self" molecule), or, for purposes of study, a nominal antigen, such as ovalbumin. The vaccine may be formulated using a variety of preparative methods known to those of skill in the art. See Remington's Pharmaceutical Sciences, Mack Publishing Co., (2000), which is incorporated herein by reference.

15        In some embodiments, antigens for inclusion in compositions of the invention are suitably derived from attenuated or killed infectious agents. It will be understood that whole microorganisms or portions thereof (e.g., membrane ghosts; crude membrane preparations, lysates and other preparations of microorganisms) may suitably be included as an antigen. Suitable infectious agents from which an antigen 20 may be derived include, but are not limited to, pathogenic viruses and microorganisms. In some contexts, suitable antigens are obtained or derived from a viral pathogen that is associated with human disease including, but not limited to, HIV/AIDS (*Retroviridae*, e.g., gp120 molecules for HIV-1 and HIV-2 isolates, HTLV-I, HTLV-11), influenza viruses (*Orthomyxoviridae*, e.g., types A, B and C), herpes (e.g., 25 herpes simplex viruses, HSV-1 and HSV-2 glycoproteins gB, gD and gH), rotavirus infections (*Reoviridae*), respiratory infections (parainfluenza and respiratory syncytial viruses), Poliomyelitis (*Picornaviridae*, e.g., polioviruses, rhinoviruses), measles and

mumps (*Paramyxoviridae*), Rubella (*Togaviridae*, e.g., rubella virus), hepatitis (e.g., hepatitis viruses types A, B, C, D, E and/or G), cytomegalovirus (e.g., gB and gH), gastroenteritis (*Caliciviridae*), Yellow and West Nile fever (*Flaviviridae*), Rabies (*Rhabdoviridae*), Korean hemorrhagic fever (*Bunyaviridae*), Venezuelan fever (5 *Arenaviridae*), warts (*Papillomavirus*), simian immunodeficiency virus, encephalitis virus, varicella zoster virus, Epstein-Barr virus, and other virus families, including *Coronaviridae*, *Birnaviridae* and *Filoviridae*.

Suitable bacterial and parasitic antigens can also be obtained or derived from known bacterial agents responsible for diseases including, but not limited to, 10 diphtheria, pertussis, tetanus, tuberculosis, bacterial or fungal pneumonia, otitis media, gonorrhea, cholera, typhoid, meningitis, mononucleosis, plague, shigellosis or salmonellosis, Legionnaires' disease, Lyme disease, leprosy, malaria, hookworm, Onchocerciasis, Schistosomiasis, Trypanosomiasis, Leishmaniasis, giardiasis, amoebiasis, filariasis, Borrelia, and trichinosis. Still further antigens can be obtained 15 or derived from unconventional pathogens such as the causative agents of kuru, Creutzfeldt-Jakob disease (CJD), scrapie, transmissible mink encephalopathy, and chronic wasting diseases, or from proteinaceous infectious particles such as prions that are associated with mad cow disease.

Specific pathogens from which antigens can be derived include *M. tuberculosis*, *Chlamydia*, *N. gonorrhoeae*, *Shigella*, *Salmonella*, *Vibrio cholerae*, 20 *Treponema pallidum*, *Pseudomonas*, *Bordetella pertussis*, *Brucella*, *Francisella tularensis*, *Helicobacter pylori*, *Leptospira interrogans*, *Legionella pneumophila*, *Yersinia pestis*, *Streptococcus* (types A and B), *pneumococcus*, *meningococcus*, *Haemophilus influenza* (type b), *Toxoplasma gondii*, *Moraxella catarrhalis*, 25 *donovanosis*, and *actinomycosis*; fungal pathogens include *candidiasis* and *aspergillosis*; parasitic pathogens include *Taenia*, *flukes*, *roundworms*, *amebiasis*, *giardiasis*, *Cryptosporidium*, *Schistosoma*, *Pneumocystis carinii*, *trichomoniasis* and

trichinosis. The present invention can also be used to provide a suitable immune response against numerous veterinary diseases, such as foot-and-mouth diseases, coronavirus, *Pasteurella multocida*, *Helicobacter*, *Strongylus vulgaris*, *Actinobacillus pleuropneumonia*, Bovine Viral Diarrhea Virus (BVDV), *Klebsiella pneumoniae*, *E. coli*, and *Bordetella pertussis*, *parapertussis* and *brochiseptica*.

5 In some embodiments, antigens for inclusion in compositions of the invention are suitably tumor-derived antigens or autologous or allogeneic whole tumor cells. Suitably, the tumor antigen is a tumor specific antigen (TSA) or a tumor associated antigen (TAA). Several tumor antigens and their expression patterns are known in 10 the art and can be selected based on the tumor type to be treated. Non-limiting examples of tumor antigens include cdk4 (melanoma),  $\beta$ -catenin (melanoma), caspase-8 (squamous cell carcinoma), MAGE-1 and MAGE-3 (melanoma, breast, glioma), tyrosinase (melanoma), surface Ig idioype (e.g., BCR) (lymphoma), Her-2/neu (breast, ovarian), MUC-1 (breast, pancreatic) and HPV E6 and E7 (cervical 15 carcinoma). Additional suitable tumor antigens include prostate specific antigen (PSA), sialyl Tn (STn), heat shock proteins and associated tumor peptides (e.g., gp96), ganglioside molecules (e.g., GM2, GD2, and GD3), Carcinoembryonic antigen (CEA) and MART-1.

## 20 Methods of stimulating NKT cells

“Stimulating an NKT cell” and “activating an NKT cell” are used interchangeably herein to refer to inducing an observable effect in an NKT cell that is consistent with a cellular response to engagement of the TCR of the NKT cell with an antigen presented in the context of CD1d. Observable effects of stimulation of NKT 25 cells include secretion of cytokines, clonal proliferation and upregulation of expression of cell surface markers, for example, CD69 molecules, IL-12 receptors and/or CD40L molecules. To stimulate an NKT cell in accordance with the present

methods, the NKT cell is contacted with a NKT cell agonist compound in an amount sufficient to induce any of the above-listed observable effects.

As used herein, "contacting an NKT cell" refers to the *in vitro* addition of an NKT cell agonist compound to NKT cells in culture, optionally in the presence of 5 immobilized, soluble, or insoluble CD1d molecules or APCs expressing CD1d molecules, or to the *in vivo* administration of NKT cell agonist compound to a subject. The NKT cell agonist compound may be presented to the TCR of the NKT cell by CD1d molecules on the surface of an antigen presenting cell (APC), such as a dendritic cell (DC). Alternatively, CD1d molecules may be plated and the NKT cells 10 and NKT cell agonist compound can be added to the CD1d molecules *in vitro*. In some *in vitro* embodiments, a lipid transfer molecule, e.g., saposin B, may be used to facilitate loading of CD1d molecules with NKT cell agonist compounds.

Examples of cytokines that may be secreted by NKT cells stimulated in accordance with the invention may include, but are not limited to, IL-10, IL-4, and IL-15, 12, IL-13, GM-CSF, IFN- $\gamma$ , IL-2, IL-1, IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$ . It is appreciated that combinations of any of the above-noted cytokines may be secreted by NKT cells upon activation. Methods for detecting and measuring levels of secreted cytokines are well-known in the art.

NKT cell proliferation may also be induced upon stimulation by contact with 20 NKT cell agonist compounds. Proliferation is suitably measured *in vitro* by standard methods, e.g.  $^3$ H-thymidine or BrdU incorporation assays.

Upregulation of cell surface markers is also suitably observed upon activation of NKT cells. For example, CD69, CD25, CD40L and IL-12 receptors are upregulated upon activation of NKT cells. Immunologic methods, such as FACS, 25 may be used to detect upregulation of cell surface markers, as well as other methods commonly employed in the art. Downstream effects of NKT cell activation, such as

induction of DC maturation, are also observable, e.g., by measuring upregulation of CD80 and/or CD86 on DCs.

*In vivo* and *ex vivo* activation of NKT cells is specifically contemplated in addition to *in vitro* activation. Presentation of NKT cell agonist compounds to NKT 5 cells results in NKT cell activation and dendritic cell maturation. Consequently, these compounds stimulate immune responses against nominal antigens as well as infectious agents and neoplastic malignancies, including solid and hematologic tumors. Both cellular and humoral immunity may be stimulated by administering NKT cell agonist compounds.

10 Methods of stimulating an NKT cell *in vivo*, i.e., in a subject, include administering a NKT cell agonist compound to the subject. Administration to a subject in accordance with some methods of the invention may include first formulating the NKT cell agonist compound with a physiologically acceptable vehicle and/or excipient to provide desired dosages, stability, etc. Suitable formulations for 15 vaccine preparations and therapeutic compounds are known in the art.

Methods of stimulating an NKT cell *ex vivo* may include use of adoptive transfer methods based on administering cells that have been contacted with NKT cell agonist compounds *ex vivo* to stimulate NKT cells in a subject. In some embodiments, the cells may be NKT cells that are stimulated *ex vivo* and injected 20 into a subject. In other embodiments, the cells may be APCs that have been contacted with NKT cell agonist compounds *ex vivo* to allow loading of the CD1d molecules with the NKT cell agonist compound for presentation to NKT cells. The *ex vivo* stimulated NKT cells or loaded APCs can then be administered, e.g., by injection into the subject.

### Methods of enhancing an immune response

Some embodiments of the invention provide a method of enhancing an immune response in a subject. A "subject" is a vertebrate, suitably a mammal, more suitably a human. As will be appreciated, for purposes of study, the subject is 5 suitably an animal model, e.g., a mouse. "Enhancing an immune response" includes, but is not limited to, inducing a therapeutic or prophylactic effect that is mediated by the immune system of the subject. More specifically, enhancing an immune response in the context of the invention refers to eliciting an NKT cell response in a subject by administering an NKT cell agonist compound, thereby inducing 10 downstream effects such as production of antibodies, antibody heavy chain class switching, maturation of APCs, and stimulation of cytolytic T cells, T helper cells and both T and B memory cells.

In some embodiments, the immune response enhanced according to the invention may be an antimicrobial immune response. Such an immune response 15 suitably promotes clearance of an infectious agent or permits immune control of the agent such that disease symptoms are reduced or resolved, e.g., a persistent or latent infection.

In other embodiments, the enhanced immune response may be an anticancer or antitumor immune response. Such an immune response suitably promotes tumor 20 rejection, reduces tumor volume, reduces tumor burden, prevents metastasis, and/or prevents recurrence of the tumor. The tumor may be any solid or hematologic tumor, including but not limited to leukemia, lymphoma, AIDS-related cancers, cancers of the bone, brain, breast, gastrointestinal system, endocrine system, eye, genitourinary tract, germ cells, reproductive organs, head and neck, musculoskeletal system, skin, 25 nervous system or respiratory system. As is appreciated in the art, a cancer-specific immune response may be monitored by several methods, including: 1) measuring cytotoxicity of effector cells, using, e.g., a chromium release assay; 2) measuring

cytokine secretion by effector cells; 3) evaluating T cell receptor (TCR) specificities, e.g., by using MHC-peptide multimers; 4) measuring the clonal composition of the T cell response; and/or 5) measuring T cell degranulation.

An enhanced immune response is also suitably assessed by the assays 5 described in the examples below. In particular, the examples demonstrate that in some embodiments, NKT cell agonist compounds are capable of activating NKT cells, inducing cytokine production, inducing maturation of APCs, enhancing cytolytic and helper T cell functions, enhancing CD8+ and CD4+ T cell recruitment, enhancing antibody production, inducing antibody class switching and breaking tolerance.

10 Enhancing an immune response in a subject in accordance with the invention may be accomplished by administering to the subject a composition including an NKT cell agonist compound and in some embodiments, an antigen. The NKT cell agonist compound and the antigen may or may not induce a detectably enhanced immune response when administered to a subject independently. However, in 15 accordance with the invention, co-administration of an NKT cell agonist compound and an antigen leads to an enhanced immune response in vaccinated or treated subjects as compared to unvaccinated or untreated subjects.

Suitably, the NKT cell agonist compound and the antigen are co-administered. The term "co-administration" is meant to refer to any administration 20 protocol in which a NKT cell agonist compound and an antigen are administered to a subject. The NKT cell agonist compound and the antigen may be in the same dosage formulations or separate formulations. Where the NKT cell agonist compound and antigen are in separate dosage formulations, they can be administered concurrently, simultaneously or sequentially (i.e., administration of one 25 may directly follow administration of the other or they may be given episodically, i.e., one can be given at one time followed by the other at a later time, e.g., within a week), as long as they are given in a manner sufficient to allow both to achieve

therapeutically or prophylactically effective amounts in the subject. The NKT cell agonist compound and the antigen may also be administered by different routes, e.g., one may be administered intravenously while the second is administered intramuscularly, intravenously or orally.

5        In some embodiments, the NKT cell agonist compound is suitably added to a vaccine composition or is co-administered with a vaccine composition. Addition of an NKT cell agonist compound to a vaccine composition or co-administration with a vaccine composition may be particularly suitable in cases where the antigen has a low rate of efficacy as a vaccine and/or must be administered in an amount or at a 10 dose greater than what might be considered ideal due to side effects, cost and/or availability of the antigen, etc. Examples of such vaccines may include, but are not limited to human papillomavirus vaccines, acute otitis media vaccine (PREVNAR®), influenza vaccines, cholera vaccines and the telomerase cancer vaccine.

15      Administration to a subject may be carried out by any suitable method, including intraperitoneal, intravenous, intramuscular, subcutaneous, transcutaneous, oral, nasopharyngeal or transmucosal absorption, among others. Suitably, the NKT cell agonist compound is administered in an amount effective to activate an NKT cell or cells such that a prophylactic or therapeutic effect is achieved in the subject, e.g., an antitumor immune response or antimicrobial immune response.

20      Administration to a subject also includes use of adoptive transfer methods based on administering cells that have been contacted with NKT cell agonist compounds *ex vivo* to stimulate or enhance an immune response in a subject. In some embodiments, the cells may be NKT cells that are activated *ex vivo* and injected into a subject to provide or enhance an immune response to, e.g., cancerous 25 cells or infectious agents. In some embodiments, the cells may be APCs that have been contacted with NKT cell agonist compounds *ex vivo* to allow complexing with the CD1d molecules expressed by the APC. Antigen presenting cells can then be

administered, e.g., by injection into the subject, to provide a suitable immune response. This method of administration allows for stimulation of the immune response with minimal exposure of the subject or the subject's cells to the NKT cell agonist compounds.

5         Administration of NKT cell agonist compounds to a subject in accordance with the invention appears to exhibit beneficial effects in a dose-dependent manner. Thus, within broad limits, administration of larger quantities of NKT cell agonist compounds is expected to activate greater numbers of NKT cells or activate NKT cells to a greater degree than does administration of a smaller amount. Moreover, 10         efficacy is also contemplated at dosages below the level at which toxicity is seen. Further, in practice, higher doses are generally used where the therapeutic treatment of a disease state is the desired end, while the lower doses are generally used for prophylactic purposes.

It will be appreciated that the specific dosage administered in any given case 15         will be adjusted in accordance with the specific NKT cell agonist compound or compounds being administered, the disease to be treated or prevented, the condition of the subject, and other relevant medical factors that may modify the activity of the NKT cell agonist compound or the response of the subject, as is well known by those skilled in the art. For example, the specific dose for a particular patient depends on 20         age, body weight, general state of health, diet, the timing and mode of administration, the rate of excretion, medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of 25         the differential activities of the NKT cell agonist compound and of a known agent such as  $\alpha$ GalCer, such as by means of an appropriate conventional pharmacological or prophylactic protocol.

The maximal dosage for a subject is the highest dosage that does not cause undesirable or intolerable side effects. The number of variables in regard to an individual prophylactic or treatment regimen is large, and a considerable range of doses is expected. It is anticipated that dosages of NKT cell agonist compound in accordance with the present invention will prevent or reduce symptoms at least 50% compared to pre-treatment symptoms. It is specifically contemplated that vaccine preparations and compositions of the invention may palliate or alleviate symptoms of the disease without providing a cure, or, in some embodiments, may be used to cure or prevent the disease or disorder.

10 Suitable effective dosage amounts for administering NKT cell agonist compounds may be determined by those of skill in the art, but typically range from about 1 microgram to about 10,000 micrograms per kilogram of body weight weekly, although they are typically about 1,000 micrograms or less per kilogram of body weight weekly. In some embodiments, the effective dosage amount ranges from about 10 to about 5,000 micrograms per kilogram of body weight weekly. In another embodiment, the effective dosage amount ranges from about 50 to about 1,000 micrograms per kilogram of body weight weekly. In another embodiment, the effective dosage amount ranges from about 75 to about 500 micrograms per kilogram of body weight weekly. The effective dosage amounts described herein refer to total amounts administered, that is, if more than one NKT cell agonist compound is administered, the effective dosage amounts correspond to the total amount administered. The NKT cell agonist compound can be administered as a single weekly dose or as divided doses.

20 In some embodiments, a tumor antigen and the NKT cell agonist compound are co-administered to a subject to induce an anti-tumor immune response in the subject. Suitably, co-administration of the antigen with the NKT cell agonist

compound enhances the anti-tumor response and results in inhibition of tumor growth, reduction in tumor burden and treatment of cancer, as described above.

Administration of a vaccine preparation or composition of the invention may suitably result in therapeutic or prophylactic treatment of an infectious disease or a 5 disease related to an infectious agent. "Treating" or "treatment" of an infectious disease includes one or more of: (1) inhibiting infection, i.e. preventing the infectious agent from establishing an infection, (2) preventing spread of the infectious agent, i.e. to other areas of the subject or from one subject to another, (3) limiting disease severity, (4) preventing recurrent infections, i.e. limiting reactivation of latent or 10 persistent infections, and (5) palliating symptoms of the infectious disease.

It is specifically contemplated that any embodiment of any method or composition of the invention may be used with any other method or composition of the invention.

As used in this specification and the appended claims, the singular forms "a," 15 "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a virus" includes a mixture of two or more viruses. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

It also is specifically understood that any numerical value recited herein 20 includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application. For example, if a range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification.

25 All publications, patents and patent applications are herein expressly incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference. In case of

conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.

The following examples are provided to assist in a further understanding of the invention. The particular materials and conditions employed are intended to be 5 further illustrative of the invention and are not limiting on the reasonable scope of the appended claims.

## EXAMPLES

### Example 1. Synthesis of PBS-32

PBS-32 was synthesized as shown in FIG.1. Reagents used in the synthesis 10 were as follows (yields in parenthesis): a) TrocCl, pyridine, CH<sub>2</sub>Cl<sub>2</sub> (77%), b) TFA, H<sub>2</sub>O, c) BzCl, pyridine, DMAP (60% for two steps), d) 1) HBr, AcOH, H<sub>2</sub>O, 2) Ag<sub>2</sub>Co<sub>3</sub>, acetone, H<sub>2</sub>O (51%), e) 1) K<sub>2</sub>CO<sub>3</sub>, CCl<sub>3</sub>CN, 2) protected ceramide, BF<sub>3</sub>OEt<sub>2</sub>, MS AW<sub>300</sub>, CH<sub>2</sub>Cl<sub>2</sub> (56%), f) Zn/Cd, AcOH (67%), g) SO<sub>3</sub>-pyridine, DMF (83%), h) NaOMe, THF, MeOH (87%).

15 Starting from diacetone glucose, a trichloroethoxycarbonate ("Troc") protecting group was added, and the acetals were hydrolyzed to give the pyranose form of the sugar. The remaining hydroxyl groups were protected as benzoates, and the anomeric hydroxyl group was liberated by a two-step process in which an anomeric bromide was incorporated by hydrolysis. Coupling of the protected 20 carbohydrate with a protected ceramide was achieved using trichloroacetimidate chemistry. The Troc group was then selectively removed and the sulfate at C3" was incorporated. Deprotection with methoxide yielded PBS-32 as a white solid.

### Example 2. Loading of CD1d molecules with synthetic NKT cell agonist 25 compounds

Whether PBS-31 and PBS-32 could be efficiently loaded onto CD1d molecules was tested using a native isoelectric focusing (IEF) gel assay in the

presence and absence of saposin B. For comparison, PBS-41 and PBS-46 (sulfo- $\beta$ -galactosylceramides), PBS-36 and PBS-58 (sulfo- $\alpha$ -glucosylceramides) and PBS-35 and PBS-40 ( $\beta$ -glucosylceramides) were also evaluated in this assay. Structural formulae for these compounds are shown in FIG. 2.

5 Each 5 $\mu$ l reaction contained 2 $\mu$ M CD1d, 20 $\mu$ M lipid and 10  $\mu$ M saposin B. Reactions were incubated for 1 hour at 37°C prior to gel loading.

Loaded species are indicated by a star in FIG. 3. The lanes of FIG. 3 were loaded as follows: 1, no lipid; 2,  $\alpha$ GalCer; 3, PBS-31; 4, PBS-32; 5, no lipid; 6, iGB3; 7, PBS-40; 8, PBS-41; 9, PBS-35; 10, PBS-46; 11, PBS-58; 12, PBS-25 (positive 10 control). As indicated in FIG. 3, saposin B was required for efficient loading of CD1d molecules with all lipid species tested, with the exception of PBS-58 and PBS-25, the positive control, which were marginally loaded in the absence of saposin B. PBS-36, the phytoceramide form of PBS-58, also loaded onto CD1d in the absence of saposin B (data not shown).

15

### **Example 3. Stimulation of an NKT cell hybridoma by PBS-31 and PBS-32**

Compounds PBS-31, PBS-32, and control lipids were evaluated for their ability to stimulate a canonical CD1d-restricted V $\alpha$ 14 NKT hybridoma cell, DN32.D3 in an assay described by Lantz, et al., J. Exp. Med. 180: 1097-1106 (1994). Irradiated 20 splenocytes were pulsed with decreasing concentrations of the lipids and control compounds, and incubated with DN32.D3 cells for 24 hours. Supernatants were harvested and IL-2 release was measured using a [ $^3$ H]-thymidine incorporation assay with an IL-2 dependent cell line as described by Cantu, et al., J. Immunol. 170:4673-4682 (2003).

25 As shown in FIG. 4, splenocytes efficiently presented PBS-31 and PBS-32, and produced a typical dose response curve. Similar results were obtained when mature DCs were used as the APCs (data not shown). DCs isolated from CD1-TD

mice having a disruption of the cytoplasmic tail of CD1d were inefficient at presenting both PBS-31 and PBS-32 (data not shown). Therefore, presentation of PBS-31 and PBS-32 and concomitant stimulation of NKT cells is dependent on lysosomal targeting of CD1d.

5 To determine whether presentation of PBS-31 and PBS-32 to NKT cells was dependent on processing by DCs, a DC-free assay was used in which murine CD1d was coated on 96 well plates. PBS-31, PBS-32 and control compounds were added at various concentrations in the presence and absence of saposin B. DN32.D3 cells were added and supernatants were harvested after 24 hours and production of IL-2 10 was measured. The assay confirmed that saposin B is required for loading and further demonstrated that: 1) stimulation of NKT cells by PBS-31 and PBS-32 does not require processing by DCs; and 2) ceramide PBS-32 was consistently more potent than phytoceramide PBS-31 (data not shown).

15 **Example 4. Cytokine production by PBS-31 and PBS-32 stimulated NKT cells**

NKT cells were stimulated with PBS-32 or control compounds presented by plate-bound CD1d, splenocytes or purified mature DCs. Production of IFN- $\gamma$ , IL-4, IL-10 and GM-CSF was measured by ELISA. As shown in FIG. 5, following 24 hours incubation with splenocytes, PBS-32 induced secretion of IFN- $\gamma$ , IL-4 and GM-CSF, 20 but not IL-10 and PBS-31 induced only GM-CSF. The control lipids (glucosyl and galactosyl) were negative in the same assays. Cytokine induction was CD1d-dependent because induction was blocked by anti-CD1d antibodies and no cytokine induction was observed when DCs from CD1d<sup>-/-</sup> mice were used (data not shown).

*In vivo*, following intravenous injection of 1  $\mu$ g of positive control compound, 25  $\alpha$ GalCer, or PBS-32, IFN- $\gamma$  production in the serum of C57Bl/6 mice was measured by ELISA and was very similar for both compounds (2500 pg/ml and 1500 pg/ml at 24 hours, respectively). IL-4 was undetectable following PBS-32 injection. Also,

PBS-32 induced earlier IFN- $\gamma$  production than  $\alpha$ -GalCer. At eight hours post-injection, PBS-32 induced 600 pg/ml IFN- $\gamma$ .

**Example 5. PBS-32-CD1d tetramer binding to V $\alpha$ 14 NKT cells**

5 PBS-32-loaded CD1d tetramers, as well as control  $\alpha$ GalCer tetramers, were prepared as described in Benlagha et al., J. Exp. Med. 191: 1895-1903 (2000) incorporated herein by reference in its entirety. The resulting tetramers were used to stain a murine V $\alpha$ 14 NKT cell line. After washing, the cells were analyzed on a FACSCalibur (BD Biosciences) using FlowJo software. PBS-32-CD1d tetramer 10 staining was less intense than for CD1d- $\alpha$ GalCer tetramers but homogenous, indicating that most canonical NKT cells were stained by this reagent (data not shown).

The affinity of CD1d-PBS-32 complexes for V $\alpha$ 14/2C $\beta$  T cell receptors (TCRs) was directly measured at 25°C by SPR on a BIACore 2000 instrument. The 15 TCR was immobilized on a sensor chip and CD1d-PBS-32 complexes were injected at 10, 5, 2.5, 1.25, 0.625, 0.3125  $\mu$ M in PBS buffer. In all experiments, empty CD1d at the same concentration (10, 5, 2.5, 1.25, 0.625, 0.3125  $\mu$ M) was used as a negative control and subtracted from the corresponding experimental group. On- and off-rates were obtained by non-linear curve fitting using the 1:1 Langmuir binding 20 model and BIAevaluation 3000 software. Chi $^2$  was 0.48. As shown in FIG. 6, CD1d-PBS-32 complex affinity for the V $\alpha$ 14/2C $\beta$  TCR was found to be 0.3 mM. As previously reported, this TCR has an affinity of 30 nM for control CD1d- $\alpha$ GalCer. Cantu et al., J. Immunol. 170: 4673-82 (2003), incorporated herein by reference in its entirety.

**Example 6. Activation of human NKT cells**

The agonistic activity of PBS-31 and PBS-32 was examined for human NKT cells. A human NKT cell line was stimulated with irradiated peripheral blood lymphocytes (PBLs) or *in vitro* matured DCs in the presence of PBS-31, PBS-32 and 5 control lipid, iGB3. (Mattner et al., *Nature* 434: 525-529 (2005) incorporated herein by reference in its entirety.) After 24 hours incubation, supernatants were harvested and assayed for the presence of IL-4 and IFN- $\gamma$  by ELISA. As shown in FIG. 7, PBS-32 was able to induce IFN- $\gamma$  and IL-4 secretion. PBS-32, PBS-31 and iGB3 induced almost equivalent amounts of IL-4, but PBS-31 induced significantly less 10 IFN- $\gamma$ .

**Example 7. Induction of DC maturation *in vivo***

Mice were injected with  $\alpha$ -GalCer, PBS-31 and PBS-32 and DC differentiation was examined in splenic DCs by analyzing cell surface marker expression.

15 Three mice in each group were injected intravenously with 1  $\mu$ g lipid or vehicle alone. At 24 hours post-injection, CD1d expression was examined on splenic B cells (B220 $+$ ), macrophages (CD11b $+$ ) and DCs (CD11c $+$ ) by FACS analysis using a FACScalibur machine and FlowJo analysis software.

In each animal, CD1d expression was increased on macrophages when 20 compared to the mean fluorescence intensity of the control group (MFI going from - 20 to 200). No differences in CD1d expression were observed on any other cell subset examined.

The maturation of DCs was analyzed by measuring expression of CD40, CD80, CD86, and MHC class II on CD11c $+$ /CD8 $^{+}$  and CD11c $^{+}$ /CD8 $^{-}$  cells (FIG. 8). 25 Both subsets of DCs demonstrated a mature phenotype as determined by cell surface marker expression after injection of PBS-32, PBS-31 or  $\alpha$ GalCer. Profiles of maturation were similar for PBS-32 and  $\alpha$ GalCer. Similar to the results obtained in

the NKT cell activation assays, PBS-31 had a less pronounced effect than did PBS-32 or  $\alpha$ GalCer. This lesser potency of PBS-31 could not be overcome by increasing the doses up to 50  $\mu$ g per mouse (data not shown). PBS-32 and  $\alpha$ GalCer reached maximal induction between 0.1 and 1  $\mu$ g (data not shown).

5 Induction of DC maturation was unaffected in MyD88<sup>-/-</sup> and MyD88<sup>-/-</sup>TRIF<sup>-/-</sup> knock out animals in which the Toll-like receptor pathway is partially, or totally impaired, respectively (data not shown). The CD1d-dependence was confirmed by injecting CD1d<sup>-/-</sup> mice with the lipids. As predicted, DC maturation did not occur in CD1d<sup>-/-</sup> mice after injection with the lipids.

10

#### **Example 8. Adjuvant capability analysis**

The potential adjuvanticity of PBS-31 and PBS-32 combined with a nominal protein antigen, ovalbumin, was conducted following a protocol similar to that of Fujii et al., J. Exp. Med. 198: 267-279 (2003) and Hermans et al., J. Immunol. 171: 5140-15 5147 (2003), which are incorporated herein by reference in their entireties. Ovalbumin was injected intravenously either alone or in combination with 1  $\mu$ g of PBS-31, PBS-32 or  $\alpha$ -GalCer (PBS-57). FIG. 9 demonstrates results from a representative experiment. In the absence of working tetramers to follow MHC class II responses, only CD8+ responses and B cell responses were evaluated.

20 CD8+ T cell responses were evaluated by enumerating the H-2K<sup>b</sup>/ova<sub>257-264</sub> tetramer positive cells in peripheral blood at 7 days post-injection and are expressed as the percentage H-2K<sup>b</sup>/ova<sub>257-264</sub> positive cells in the CD8+ T cell pool. At day 7 post-injection, antigen-specific H-2K<sup>b</sup>/ova<sub>257-264</sub> tetramer positive cells were increased to about 3% in the PBS-32 injected group whereas they expanded to almost 6% in 25 the  $\alpha$ -GalCer treated group (FIG. 9A). In both groups, the IgG anti-ovalbumin response was dramatically boosted in a dose-dependent manner when compared to the control group as measured by ELISA using an anti-IgG specific secondary

antibody (FIG. 9B). These strong IgG responses may indirectly reflect a significant boost of the ovalbumin-specific CD4+ T helper compartment as well.

When CD1d<sup>-/-</sup> mice were injected following the same protocol, no increase in either anti-ovalbumin IgG or in the anti-ovalbumin CD8+ T cells were noted (data not shown). These results confirmed that PBS-32 had adjuvant activity when used in combination with a purified protein antigen.

#### **Example 9. PBS-32 recruits T-cells in an LCMV model**

The ability to use NKT cell/DC cooperation to stimulate adaptive immunity was further tested in a model infection system, the lymphocytic choriomeningitis virus (LCMV) infection of mice. The natural host of LCMV is the mouse and both anti-viral MHC class I-restricted and MHC class II-restricted T cell responses have been finely mapped. Seven epitopes are identified for the nucleoprotein (NP) and glycoprotein (GP) on H-2K<sup>b</sup> (NP<sub>205-212</sub>, GP<sub>34-43</sub>, GP<sub>118-125</sub>) and H-2 D<sup>b</sup> (NP<sub>396-404</sub>, GP<sub>33-41</sub>, GP<sub>92-101</sub>, GP<sub>276-286</sub>) and two epitopes are restricted on I-A<sup>b</sup> (GP<sub>61-80</sub>, NP<sub>309-328</sub>).

To evaluate if the injection of PBS-32 or control  $\alpha$ GalCer could change the dynamics of the anti-LCMV T cell response, C57Bl/6 mice were injected intravenously with 1  $\mu$ g of PBS-32,  $\alpha$ GalCer or vehicle before being inoculated intraperitoneally with 10<sup>6</sup> PFU of infectious LCMV<sub>Arm</sub>. At day 8 post-infection, the frequency of CD8+ T cells specific for GP<sub>33</sub>, NP<sub>396</sub> and GP<sub>276</sub>, and the frequency of CD4+ T cells specific for GP<sub>61</sub> and NP<sub>309</sub>, was determined by intracellular FACS staining for IFN- $\gamma$  after a five hour *in vitro* pulse with peptide and APC. As seen in FIG. 10, LCMV alone induced a strong anti-LCMV class I and class II restricted response against all tested epitopes (black bars). However, despite this strong basal response,  $\alpha$ -GalCer was able to significantly increase the CD8+ response (white bars), and PBS-32 did equally well (grey bars). More surprisingly, whereas the CD4+ response was unchanged by the injection of  $\alpha$ -GalCer, PBS-32 was able to induce a

significant increase of both anti-GP<sub>61</sub> and anti-NP<sub>309</sub> I-A<sup>b</sup>-restricted responses. Therefore, in contrast to the results obtained with a protein antigen,  $\alpha$ GalCer and PBS-32 have different abilities to recruit CD4+ and CD8+ T cells in this viral infection model.

5

**Example 10. PBS-32 enhances vaccination against infection with a pathogenic microorganism**

The ability of NKT cell agonist compounds to stimulate a memory immune response and vaccinate against infection with a pathogenic microorganism will be 10 tested in a mouse infection model. Mice will be injected intraperitoneally or intravenously with one of the following: a composition comprising PBS-32 and an infectious agent antigen such as lipoarabinomannan from *Mycobacterium tuberculosis* in a physiologically acceptable vehicle; the antigen alone in a physiologically acceptable vehicle; or the vehicle alone. At least one week after 15 immunization, the immune response will be assessed by harvesting the spleen, stimulating the recovered cells *in vitro* with the antigen and performing ELISA and FACS analysis to assess cytokine induction, expression of activation-related cell surface markers and antibody production on various immune cell types including NKT cells, CD4+ T cells, CD8+ T cells, APCs and B cells.

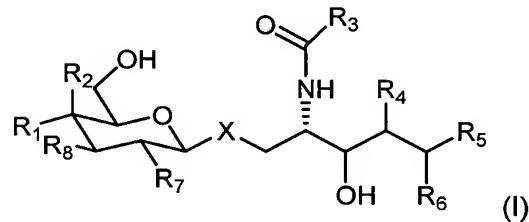
20 A second set of immunized animals will be experimentally infected with decreasing doses of the infectious agent to produce a dose response curve and determine the LD<sub>50</sub> (amount of infectious agent required to kill 50% of the animals) and ID<sub>50</sub> (amount of infectious agent required to establish an infection). The animals will be scored on a scale of 1 to 5 for disease symptom severity (1 being no disease 25 symptoms and 5 representing dead or euthanized animals). The animals will be scored on a daily basis after challenge to assess whether the immunization reduced disease severity. Sample tissues will be harvested at various time points over the

course of the infection and assayed for the presence of the infectious agent by methods known to those of skill in the art. The results will demonstrate that when the immunization procedure included PBS-32 as an adjuvant, the immune response to the antigen is enhanced, the LD<sub>50</sub> is higher and the ID<sub>50</sub> is higher (i.e., more 5 microorganisms are needed to kill or establish an infection in the animal).

The invention has been described with reference to various specific embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

We claim:

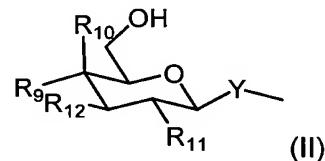
1. A composition comprising a compound represented by structural formula (I):



5 wherein:

X is -O-, -CH<sub>2</sub>- or -S-;

R<sub>1</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H, -COOH or a group represented by structural formula (II):



10 wherein:

Y is -O-, -CH<sub>2</sub>- or -S-;

R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH;

15 R<sub>2</sub> is selected from -H, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH;

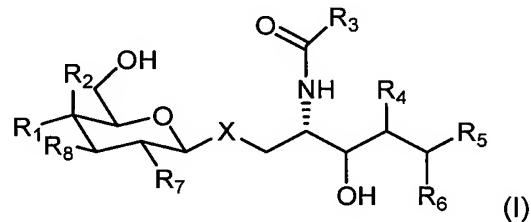
R<sub>3</sub> is a saturated or unsaturated hydrocarbon group having from about 7 to about 25 carbon atoms;

R<sub>4</sub> is -H, -OH, or, together with R<sub>6</sub>, forms a carbon-carbon double bond;

20 R<sub>5</sub> is a saturated or unsaturated hydrocarbon group having from about 5 to about 15 carbon atoms,

$R_6$  is  $-H$ ,  $-OH$ , or together with  $R_4$  forms a C-C double bond;  
 $R_7$  and  $R_8$  are independently selected from  $-H$ ,  $-OH$   $-OSO_3H$   $-SO_3H$ ,  $-PO_4$ ,  
 5  $-PO_4H$  and  $-COOH$ ;  
 provided that at least one of  $R_1$ ,  $R_2$ ,  $R_7$ ,  $R_8$ ,  $R_9$ ,  $R_{10}$ ,  $R_{11}$  or  $R_{12}$  is  $-H$ ,  $-OH$ ,  $-OSO_3H$ ,  $-SO_3H$ ,  $-PO_4$ ,  $-PO_4H$  or  $-COOH$ ; and  
 and a physiologically acceptable vehicle.

2. A composition comprising a compound represented by structural formula (I):

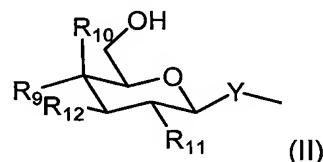


10

wherein:

 $X$  is  $-O-$ ,  $-CH_2-$  or  $-S-$ ;

$R_1$  is  $-H$ ,  $-OSO_3H$ ,  $-SO_3H$ ,  $-PO_4$ ,  $-PO_4H$ ,  $-COOH$  or a group  
 represented by structural formula (II):



15

wherein:

 $Y$  is  $-O-$ ,  $-CH_2-$  or  $-S-$ ;

20

$R_9$ ,  $R_{10}$ ,  $R_{11}$  and  $R_{12}$  are independently selected from  $-H$ ,  $-OH$ ,  $-OSO_3H$ ,  
 $-SO_3H$ ,  $-PO_4$ ,  $-PO_4H$  and  $-COOH$ ;  
 $R_2$ , is selected from  $-H$ ,  $-OH$ ,  $-OSO_3H$ ,  $-SO_3H$ ,  $-PO_4$ ,  
 $-PO_4H$  and  $-COOH$ ;

$R_3$  is a saturated or unsaturated hydrocarbon group having from about 7 to about 25 carbon atoms;

$R_4$  is -H, -OH, or, together with  $R_6$ , forms a carbon-carbon double bond;

5  $R_5$  is a saturated or unsaturated hydrocarbon group having from about 5 to about 15 carbon atoms;

$R_6$  is -H, -OH, or together with  $R_4$  forms a C-C double bond; and

$R_7$  is selected from -H, -OH,  $-\text{OSO}_3\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $-\text{PO}_4$ ,  $-\text{PO}_4\text{H}$  and  $-\text{COOH}$ ; and

10  $R_8$  is selected from -H, -OH,  $-\text{PO}_4$ ,  $-\text{PO}_4\text{H}$  and  $-\text{COOH}$ ;

provided that at least one of  $R_1$ ,  $R_2$ ,  $R_7$ ,  $R_8$ ,  $R_9$ ,  $R_{10}$ ,  $R_{11}$  or  $R_{12}$  is -H, -OH, - $\text{OSO}_3\text{H}$ ,  $-\text{SO}_3\text{H}$ ,  $-\text{PO}_4$ ,  $-\text{PO}_4\text{H}$  or  $-\text{COOH}$ ;

and a physiologically acceptable vehicle.

3. The composition of claim 1 or claim 2, formulated for *in vivo* administration.

15 4. The composition of any of claims 1 to 3, further comprising an antigen.

5. The composition of claim 4, wherein the antigen is derived from an infectious agent.

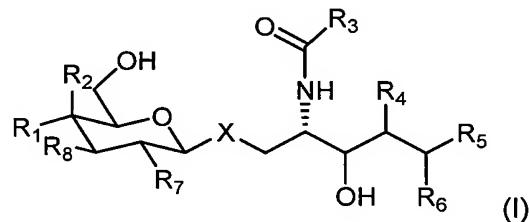
20 6. The composition of claim 4, wherein the antigen is derived from a tumor.

7. A method of enhancing an immune response in a subject comprising administering to the subject the composition of any of claims 1 to 6.

25 8. The method of claim 7, wherein the immune response comprises the production of antibodies or comprises a helper, cytolytic or memory response.

9. The composition of claim 1 or claim 2, formulated for *in vitro* application to cultured cells.

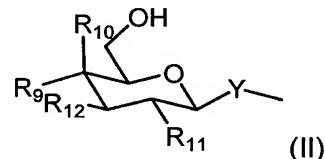
5 10. A method of stimulating an NKT cell comprising contacting the NKT cell with a compound having a structural formula represented by (I):



wherein:

10 X is -O-, -CH<sub>2</sub>- or -S-;

R<sub>1</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H, -COOH or a group represented by structural formula (II):



wherein:

15 Y is -O-, -CH<sub>2</sub>- or -S-;

R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH;

20 R<sub>2</sub> is selected from -H, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH;

R<sub>3</sub> is a saturated or unsaturated hydrocarbon group having from about 7 to about 25 carbon atoms;

$R_4$  is -H, -OH, or, together with  $R_6$ , forms a carbon-carbon double bond;

$R_5$  is a saturated or unsaturated hydrocarbon group having from about 5 to about 15 carbon atoms,

5  $R_6$  is -H, -OH, or together with  $R_4$  forms a C-C double bond; and

$R_7$  and  $R_8$  are independently selected from -H, -OH - $OSO_3H$  - $SO_3H$  -

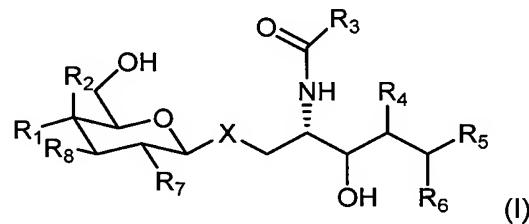
$PO_4$ ,

- $PO_4H$  and -COOH;

provided that at least one of  $R_1$ ,  $R_2$ ,  $R_7$ ,  $R_8$ ,  $R_9$ ,  $R_{10}$ ,  $R_{11}$  or  $R_{12}$  is -H, -

10 OH, - $OSO_3H$ , - $SO_3H$ , - $PO_4$ , - $PO_4H$  or -COOH.

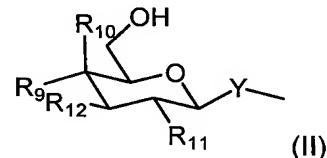
11. A method of stimulating an NKT cell comprising contacting the NKT cell with a compound having a structural formula represented by (II):



wherein:

$X$  is -O-, - $CH_2$ - or -S-;

$R_1$  is -H, - $OSO_3H$ , - $SO_3H$ , - $PO_4$ , - $PO_4H$ , -COOH or a group represented by structural formula (II):



20

wherein:

$Y$  is -O-, - $CH_2$ - or -S-;

R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are independently selected from -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>3</sub>H and -COOH;

R<sub>2</sub>, is selected from -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH;

5 R<sub>3</sub> is a saturated or unsaturated hydrocarbon group having from about 7 to about 25 carbon atoms;

R<sub>4</sub> is -H, -OH, or, together with R<sub>6</sub>, forms a carbon-carbon double bond;

10 R<sub>5</sub> is a saturated or unsaturated hydrocarbon group having from about 5 to about 15 carbon atoms;

R<sub>6</sub> is -H, -OH, or together with R<sub>4</sub> forms a C-C double bond; and

R<sub>7</sub> is selected from -H, -OH, -OSO<sub>3</sub>H, SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH; and

15 R<sub>8</sub> is selected from -H, -OH, -PO<sub>4</sub>, -PO<sub>4</sub>H and -COOH; provided that at least one of R<sub>1</sub>, R<sub>2</sub>, R<sub>7</sub>, R<sub>8</sub>, R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub> or R<sub>12</sub> is -H, -OH, -OSO<sub>3</sub>H, -SO<sub>3</sub>H, -PO<sub>4</sub>, -PO<sub>4</sub>H or -COOH.

12. The method of claim 10 or claim 11, further comprising contacting the NKT  
20 cell with the composition in the presence of a CD1d monomer or tetramer.
13. The method of claim 12, wherein the compound is bound to the CD1d monomer or tetramer.
14. The method of claim 12 or claim 13, wherein the CD1d monomer or tetramer is soluble.
- 25 15. The method of claim 12 or claim 13, wherein the CD1d monomer is expressed on a cell surface.
16. The method of claim 15, wherein the cell is an antigen presenting cell.

17. The method of any of claims 10 to 16, wherein the NKT cell is cultured *in vitro*.
18. The method of any of claims 10 to 16, wherein the NKT cell is in a subject.
19. A vaccine preparation comprising the composition of any of claims 1 to 6.
- 5 20. A vaccine preparation comprising the NKT cell of any of claims 10 to 16.

1/15

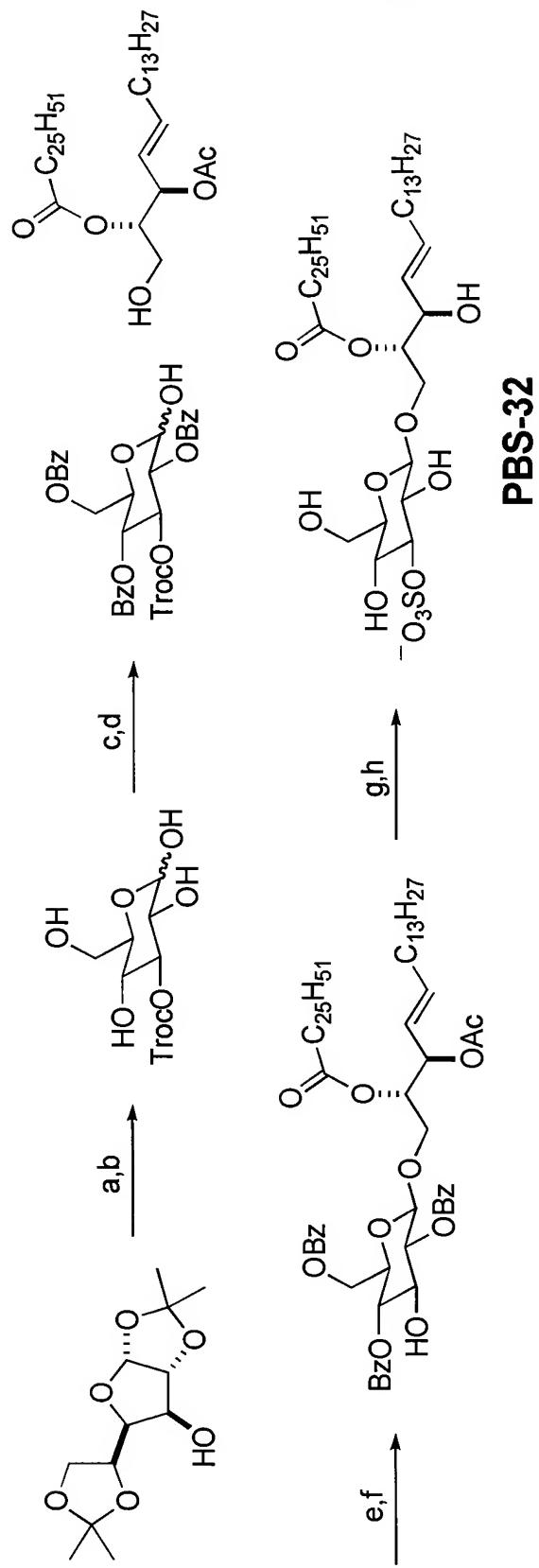
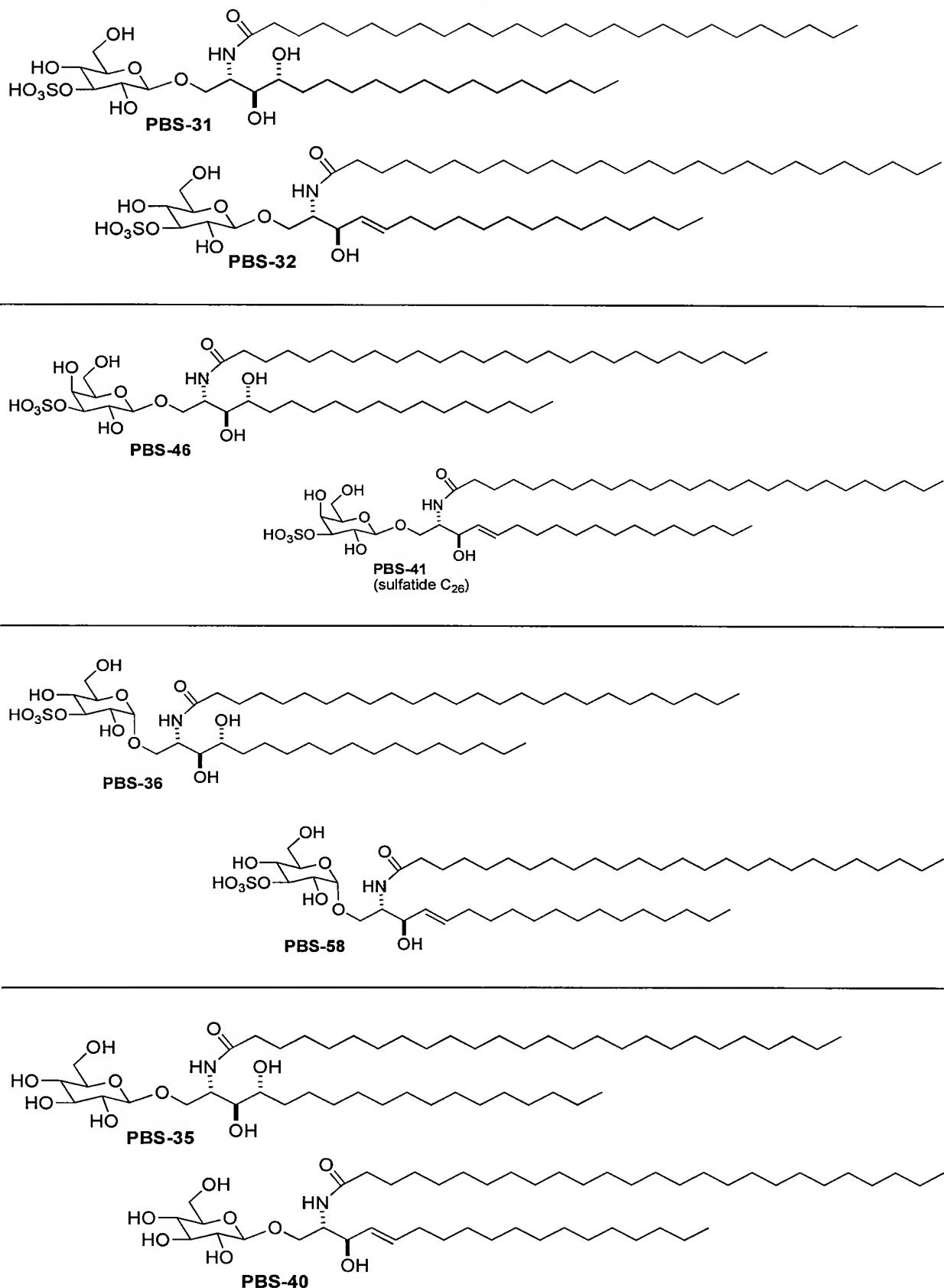
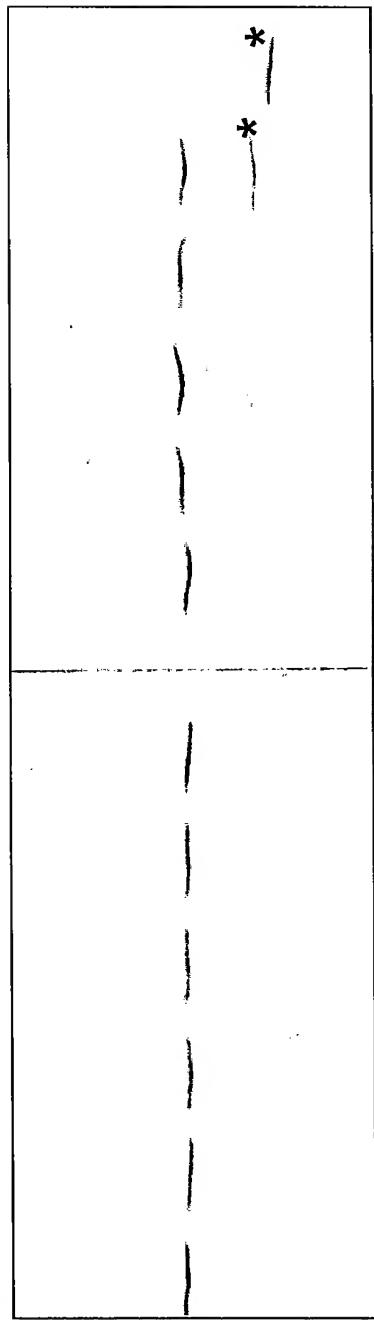


FIG. 1

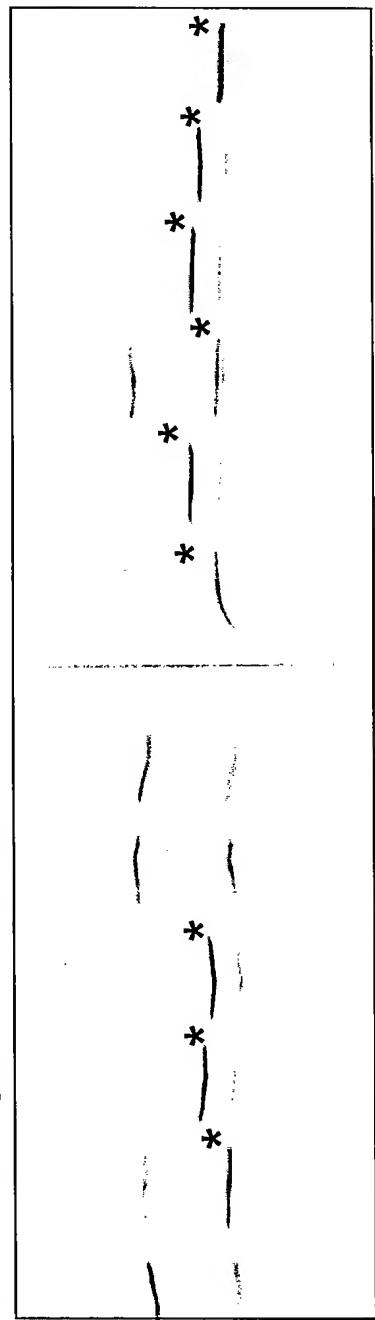
2/15

**FIG. 2**

CD1d+lipid



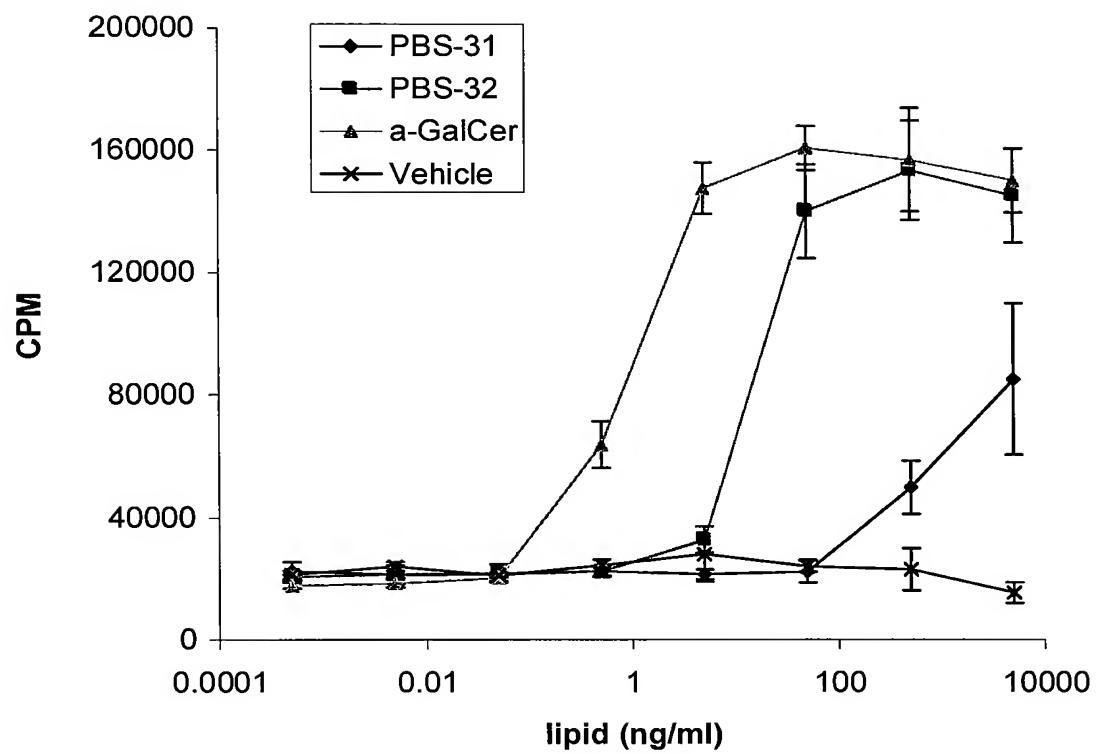
CD1d+lipid+Saposin B



1 2 3 4 5 6 7 8 9 10 11 12

FIG. 3

4/15

**FIG. 4**

5/15

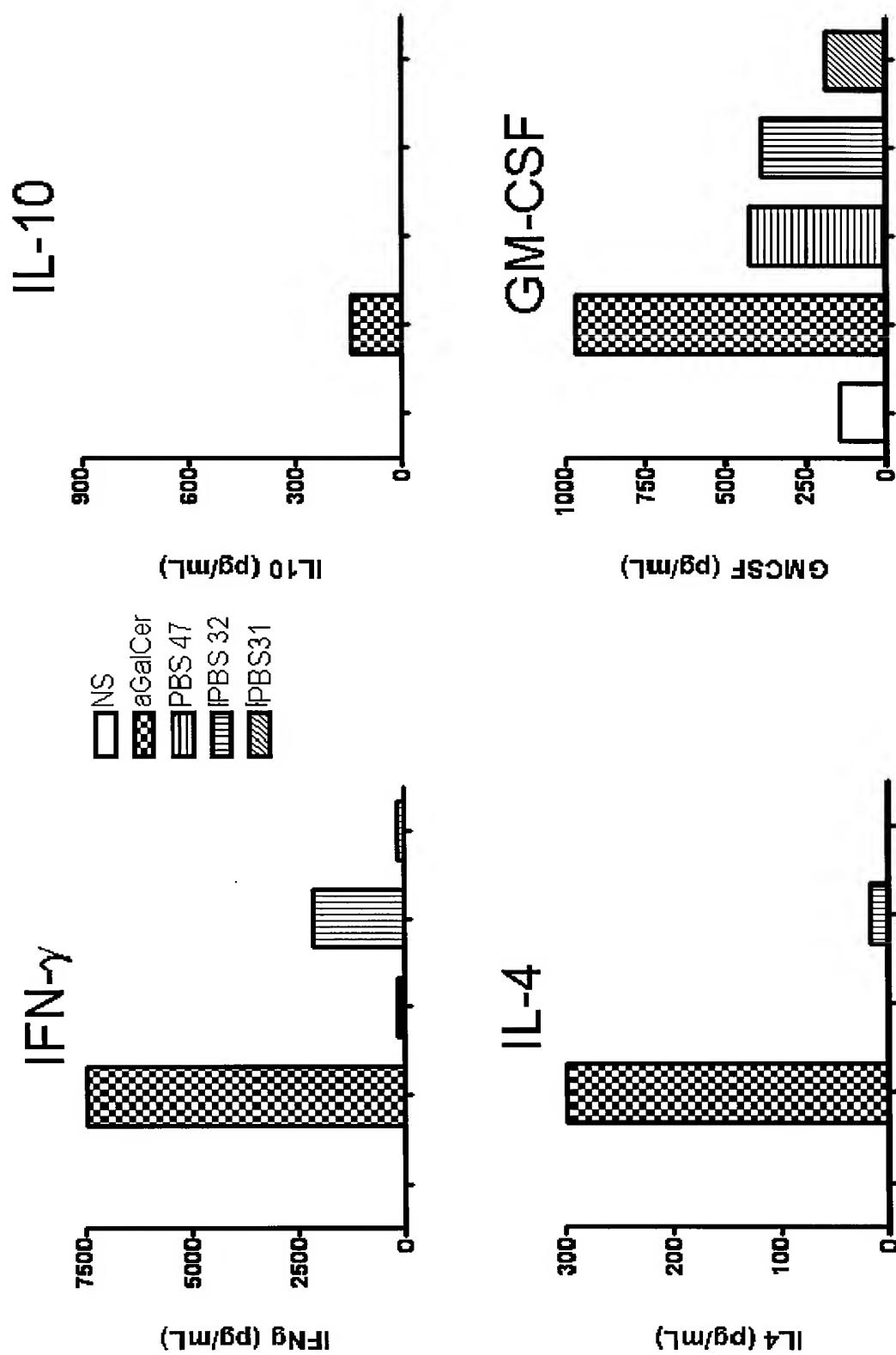
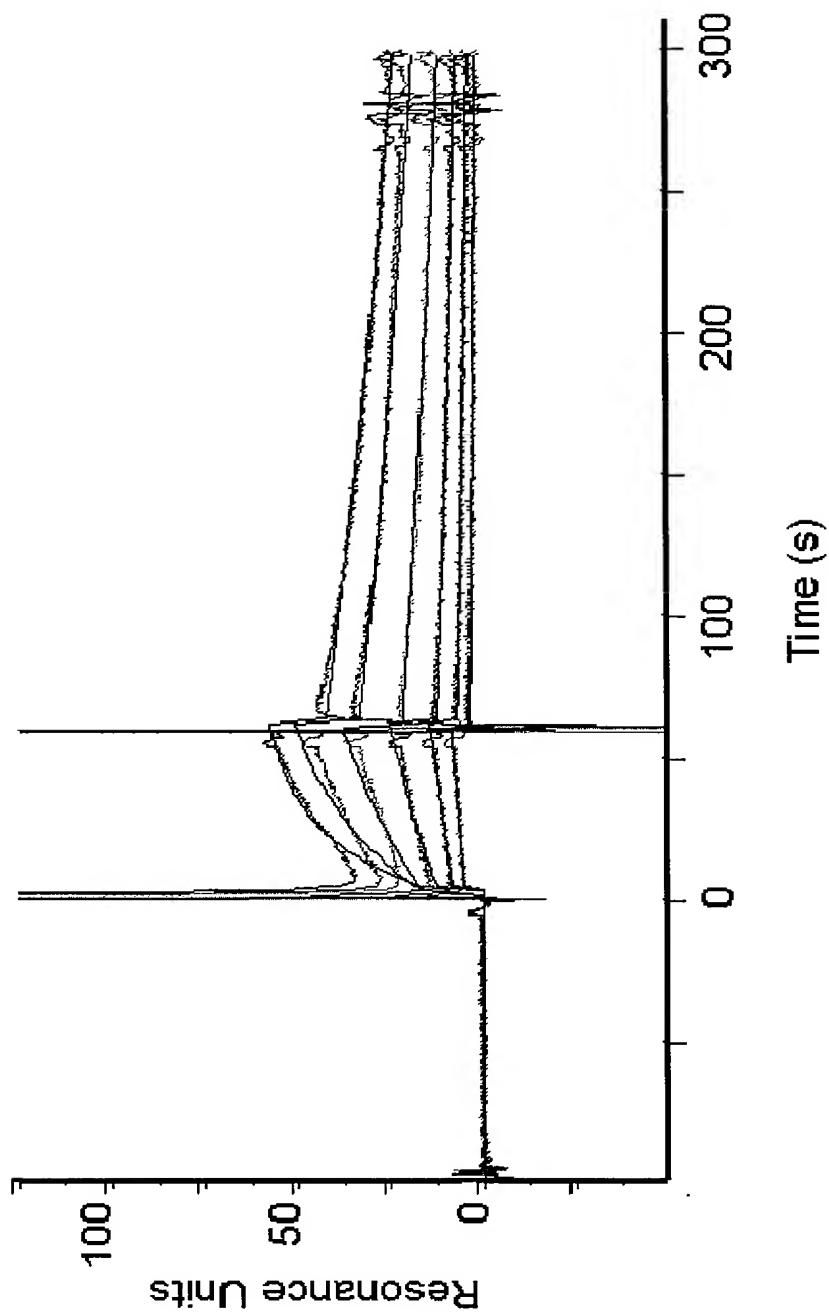
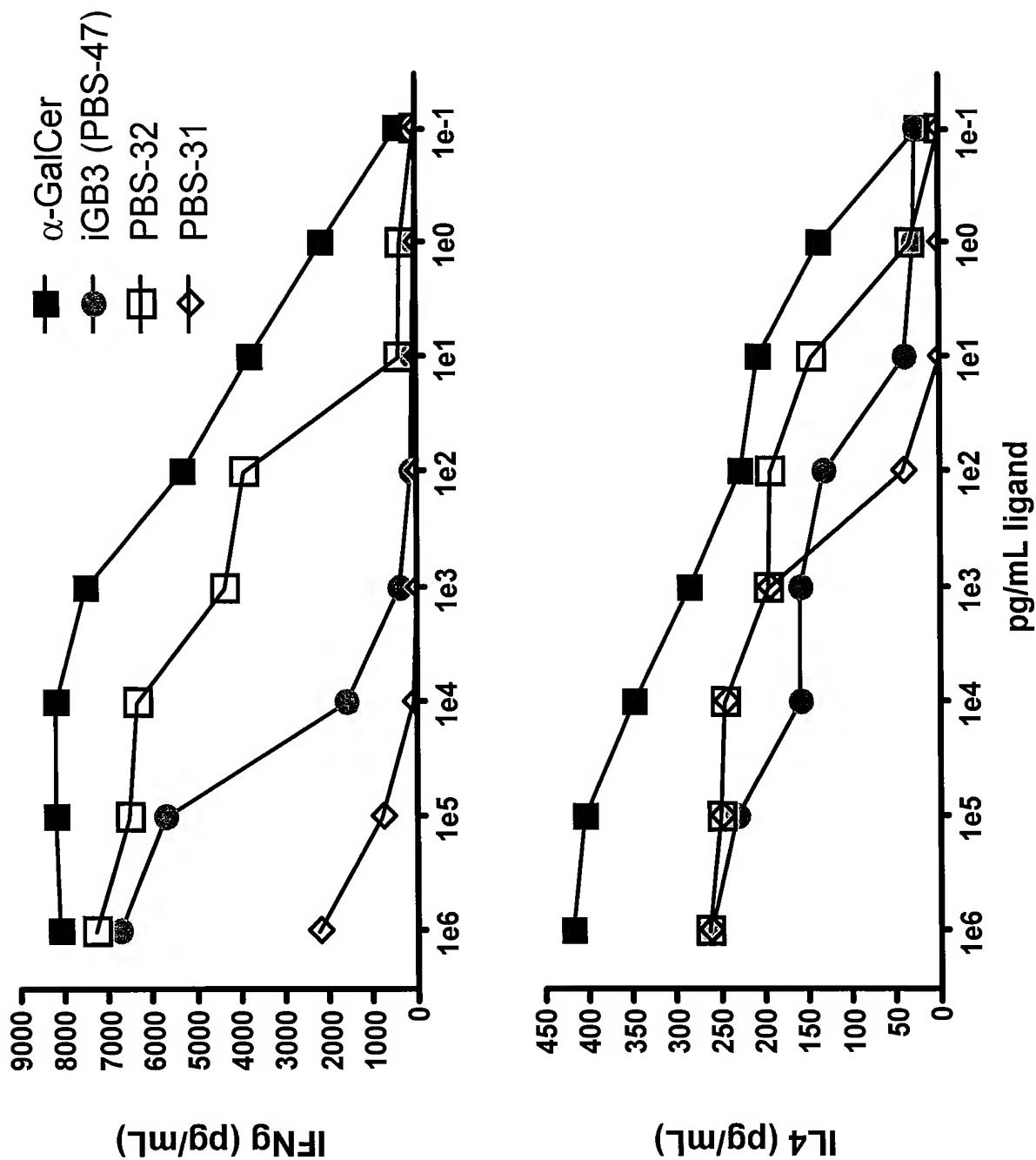


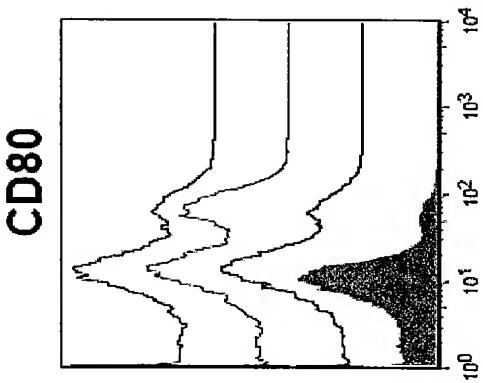
FIG. 5



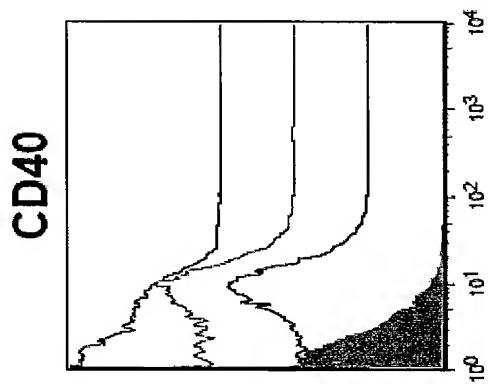
**FIG. 6**



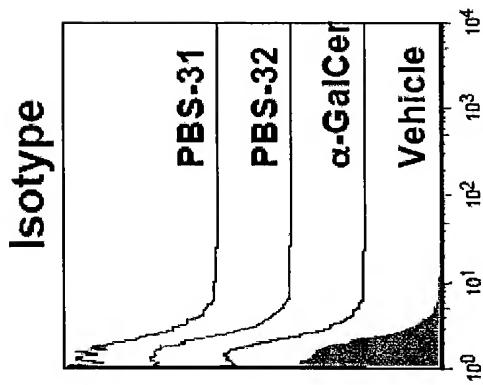
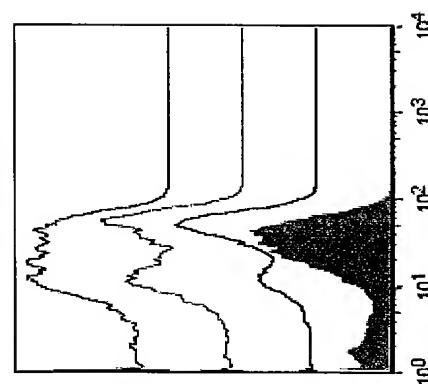
## CD11c+, CD8a-



8/15



class II



CD86

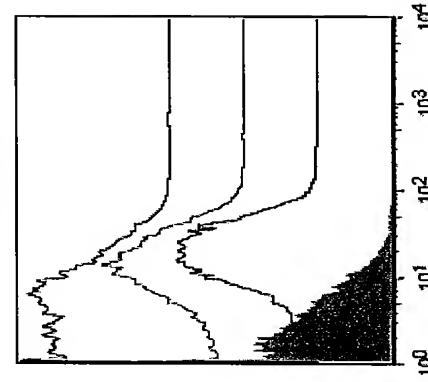
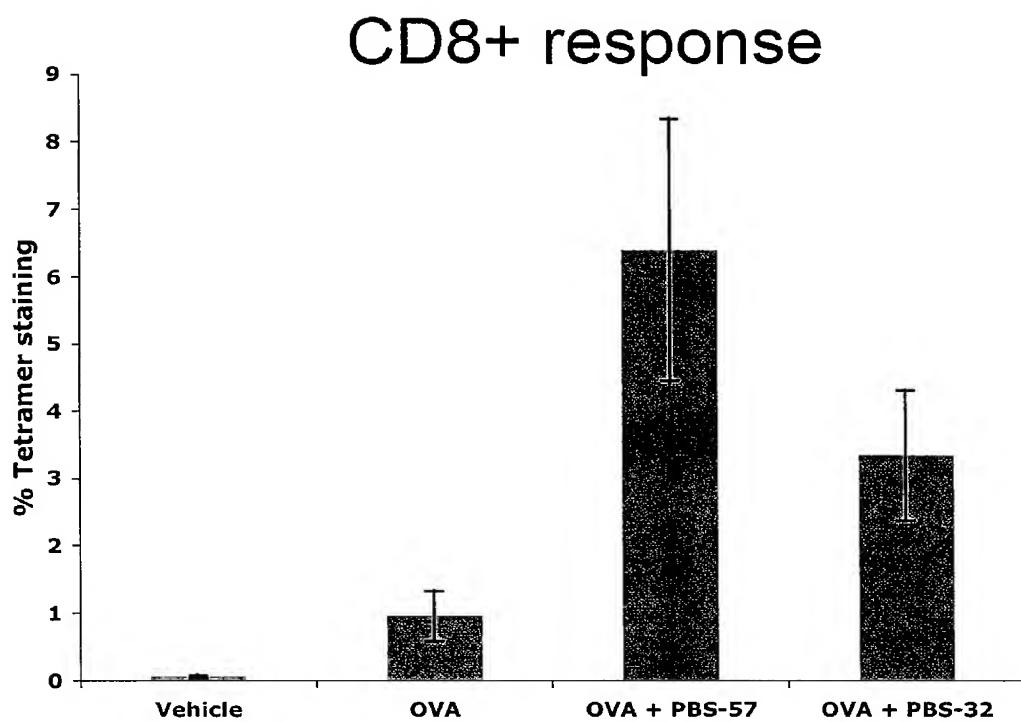
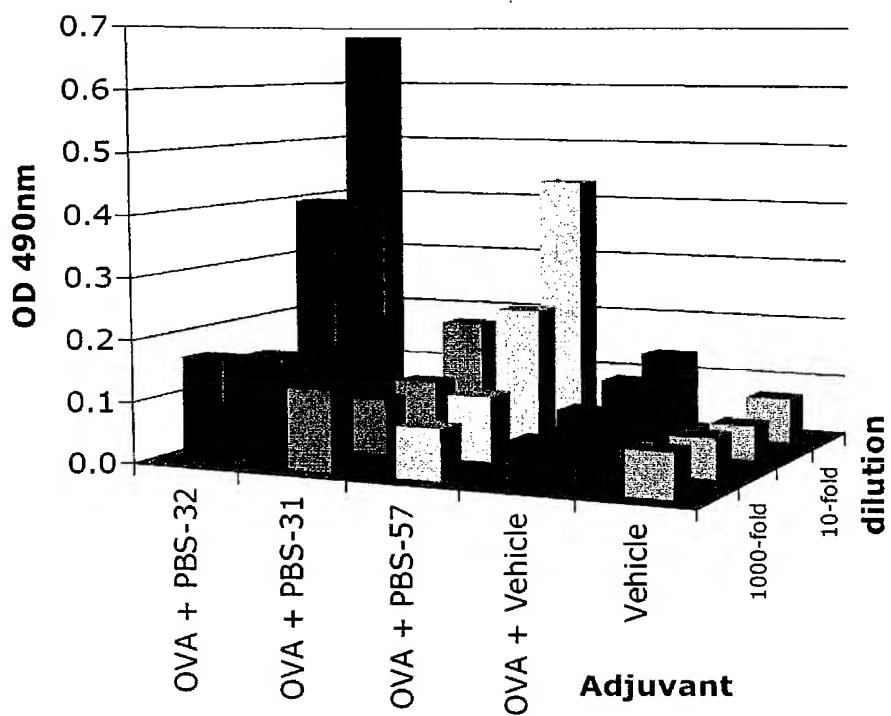


FIG. 8

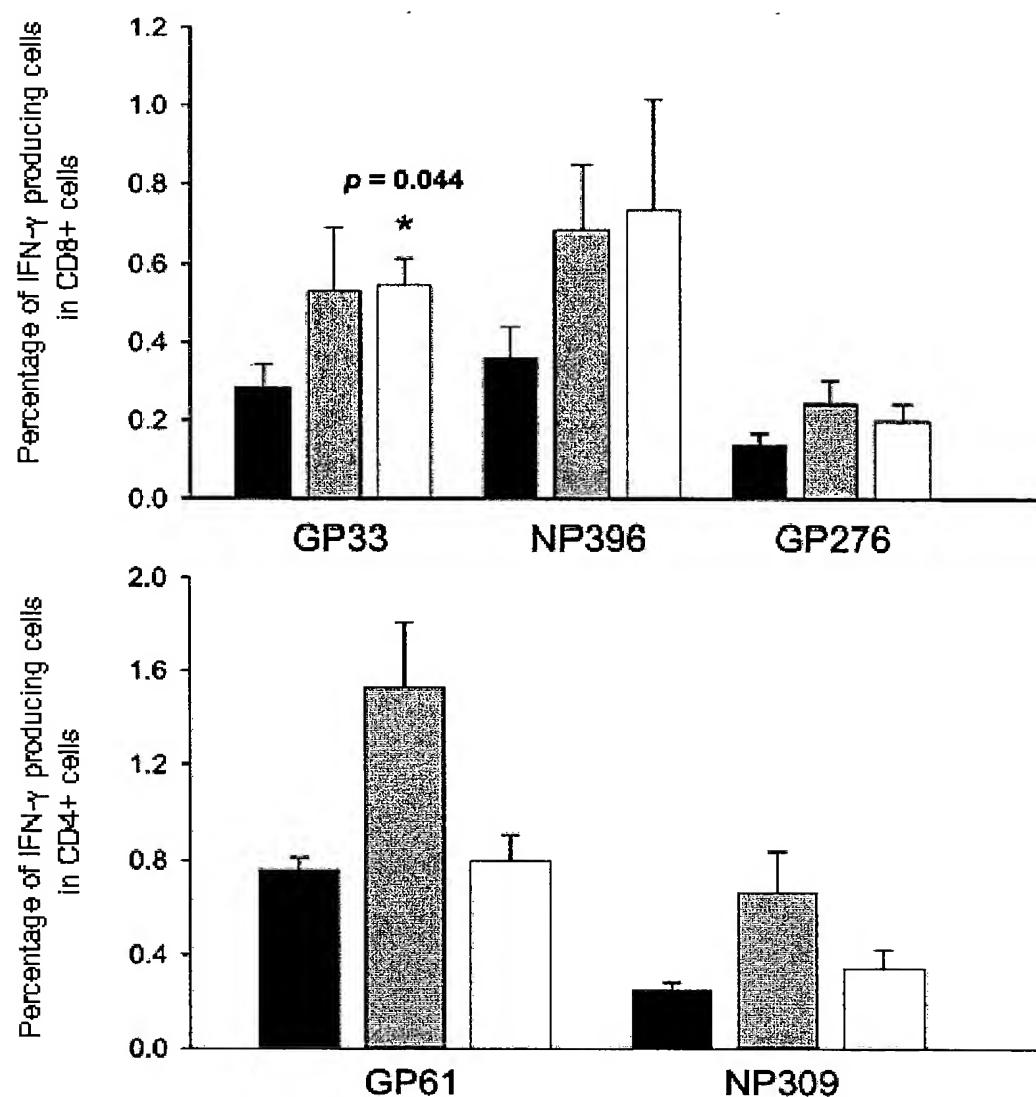
**FIG. 9A**

## Anti-ovalbumin IgG response



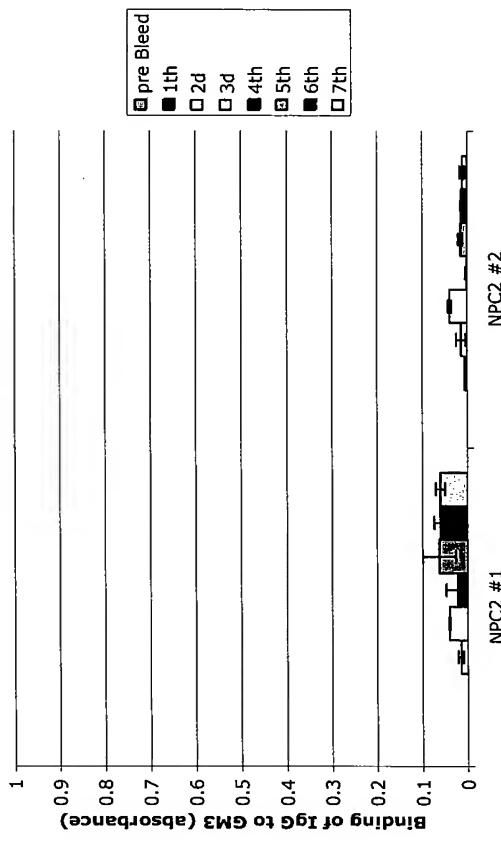
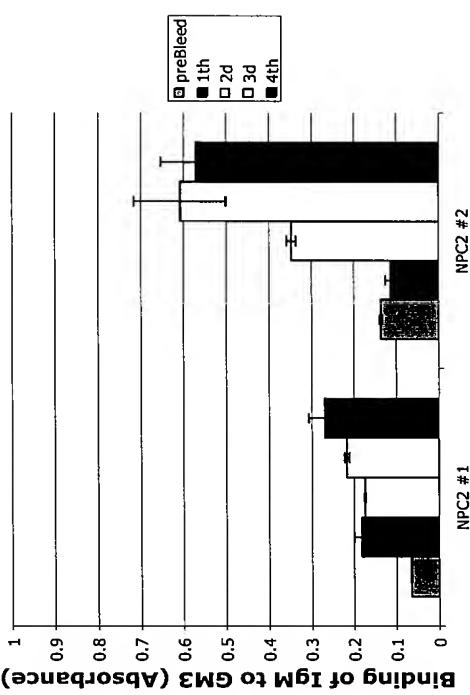
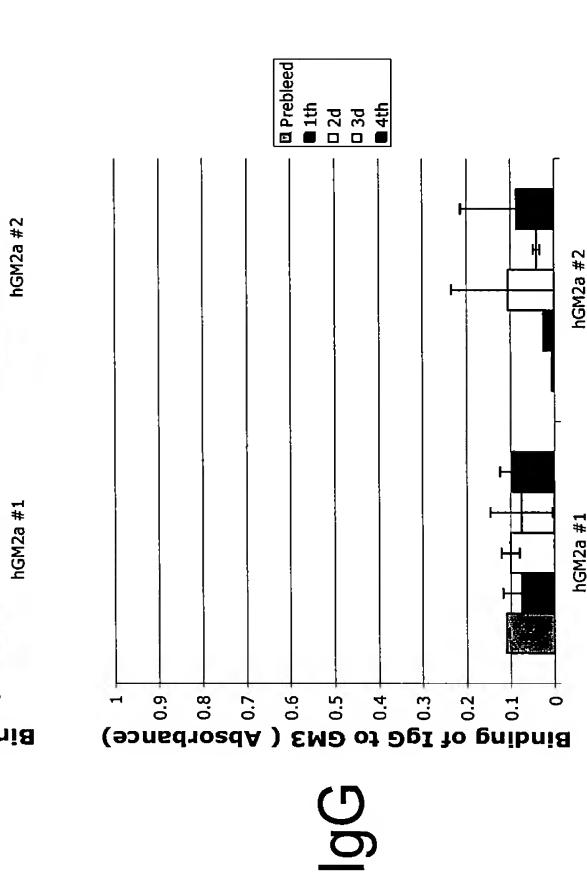
**FIG. 9B**

11/15

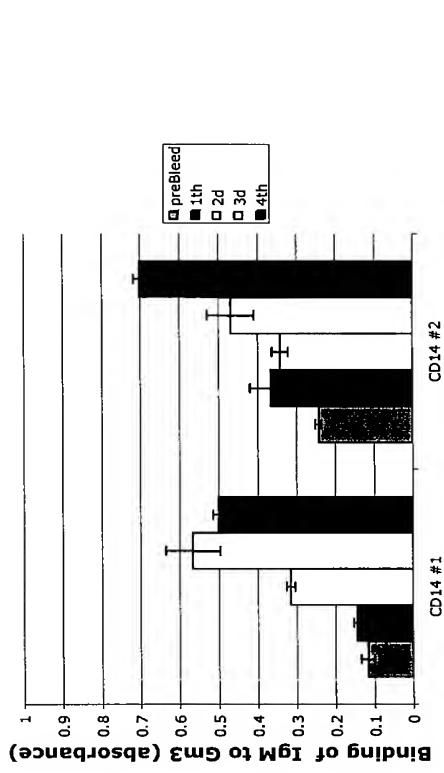
**FIG. 10**

12/15

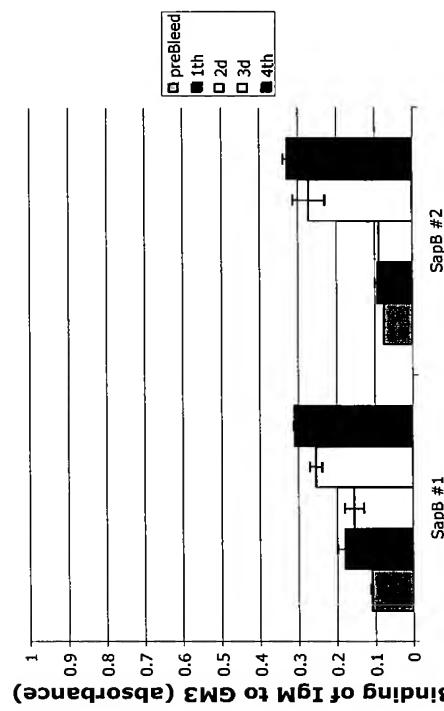
mNPC2

**FIG. 11A**

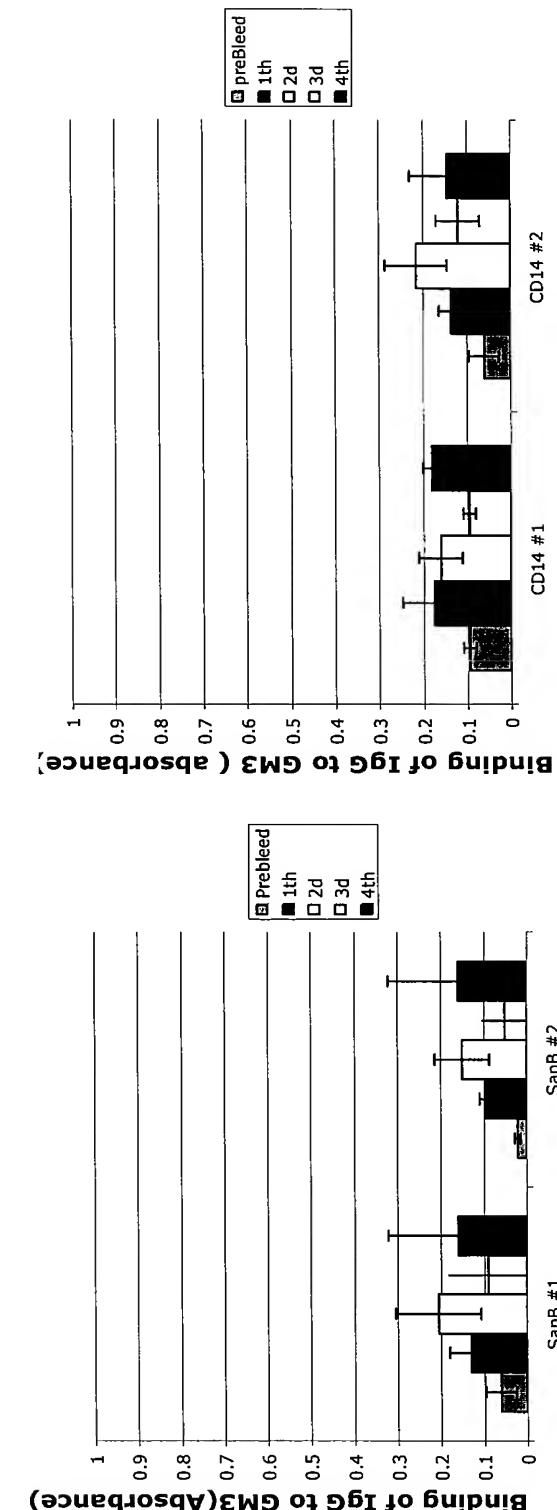
mCD14



mSapB

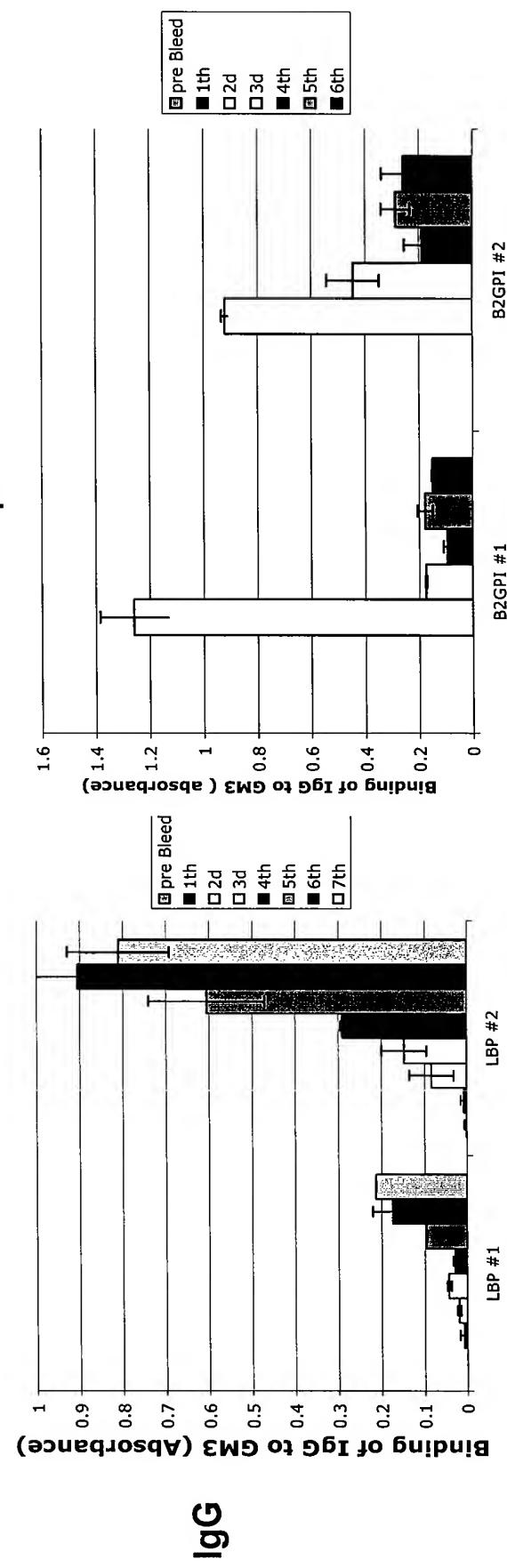
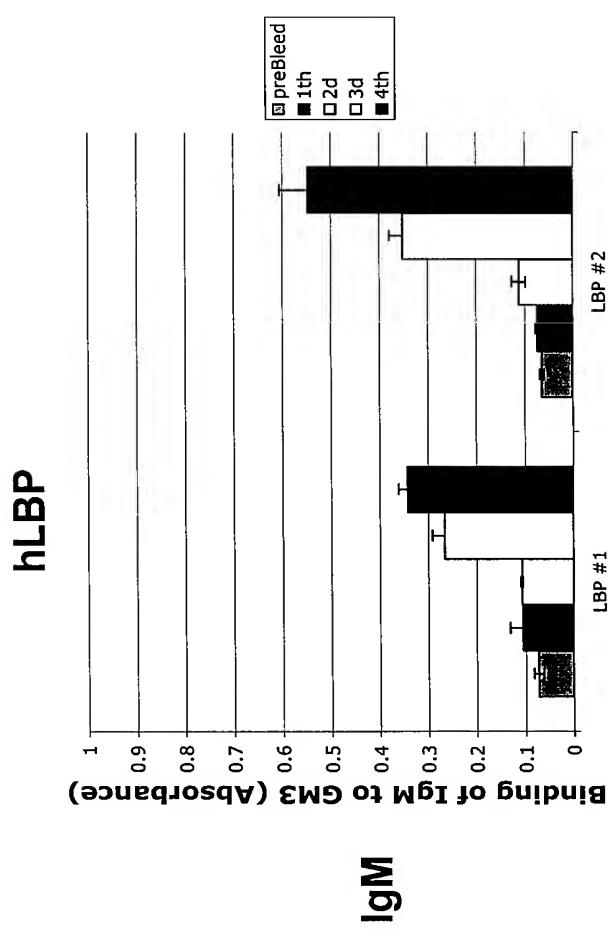


IgM

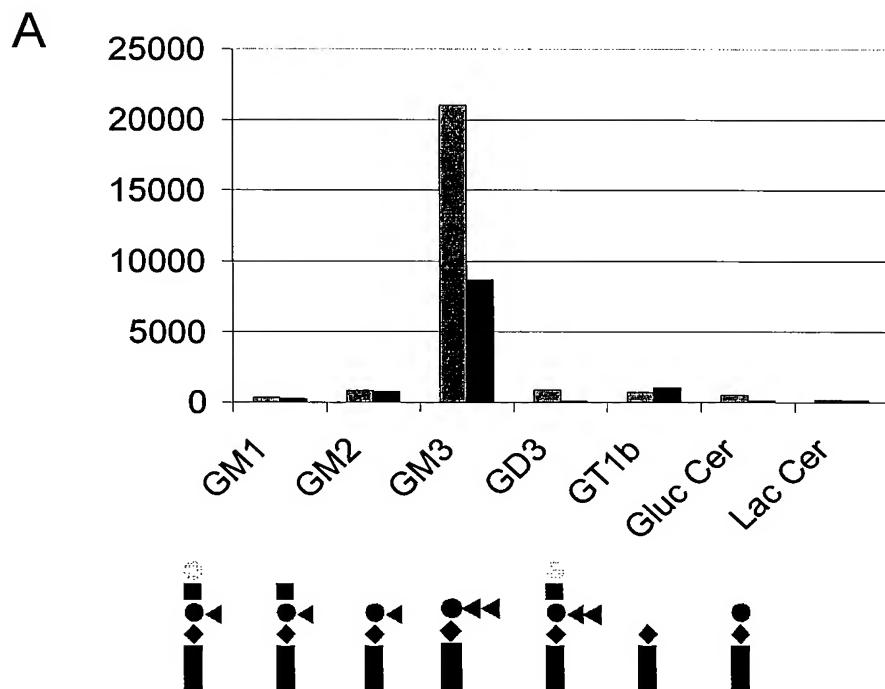
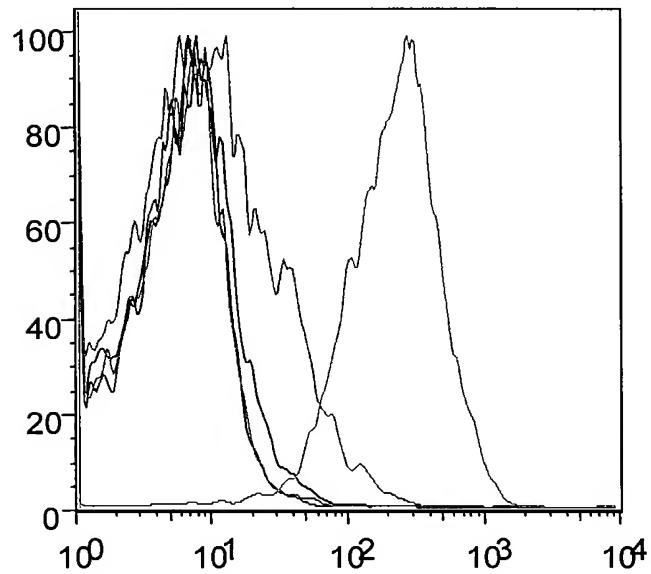


IgG

FIG. 11B

**FIG. 11C**

15/15

**B****FIG. 12**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 07/72451

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 37/00; A61K 31/20; 51/00 (2007.10)

USPC - 514/558

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC-514/558

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
USPC-424/1.73; 424/418; 424/420; 424/476; 424/498; 424/502; 514/786 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST: DB=PGPB,USPT,USOC,EPAB,JPAB

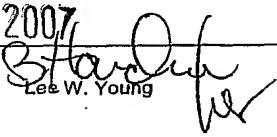
Google: NKT cells and IGB3 and CD1d

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/0073118 A1 (BENDELAC et al.) 06 April 2006 (06.04.2006) para [0007]-[0008], [0015], [0027], [0039], [0058]; pg 7, Claim 9	1-3, 9-13

 Further documents are listed in the continuation of Box C. 

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
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Date of the actual completion of the international search	Date of mailing of the international search report
19 October 2007 (19.10.2007)	27 NOV 2007 
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